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FELINE IL-12 AND IL-18 AS ADJUVANTS TO A DNA VACCINE FOR FeLV

By

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For Paul, for his constant strength, support and understanding.

SUMMARY

The Th1 cytokines interleukin 12 (IL-12) and interleukin 18 (IL-18) are important mediators of the cell-mediated immune response. Separately, both cytokines act on T and natural killer (NK) cells and stimulate the production of interferon gamma (IFN γ). These molecules can also act synergistically on their effector cells to produce a dramatic IFN γ response. IL-12 is important in the differentiation and proliferation of T helper type 1 (Th1) cells, while IL-18 increases Th1 proliferation and IL-12-driven Th1 development. The role of these interleukins in the cellular immune pathway has led to their use as adjuvants to vaccines that require strong cell mediated immunity.

A previous vaccination trial investigated the use of various feline cytokine constructs in their role as adjuvants to a DNA vaccine to feline leukaemia virus (FeLV) [Hanlon *et al.* 2001]. This study demonstrated that cytokine plasmid constructs encoding IL-12 and IL-18, together elicited complete protection from viraemia and significant protection of animals from latent proviral infection. *In vitro* analysis of these cytokine constructs prior to use *in vivo*, failed to detect IL-18 protein expression by Western blot analysis. In addition, suitable assays for measurement of *in vitro* bioactivity of these constructs were not available at that time.

The preliminary work for this project focused on the development of improved feline IL-12 and IL-18 constructs and demonstration of *in vitro* expression and bioactivity. IL-12 may be administered using separate plasmids encoding the subunits p35 and p40, which can potentially lead to several problems. Firstly cells must take up both plasmids in order to produce bioactive IL-12 heterodimer. Also the overproduction of p40 subunit is a potential risk, as p40 homodimer molecules can produce antagonism of the heterodimer by binding to the IL-12 receptor and rendering it inactive. A new construct was therefore developed linking the cDNA of each subunit by a synthetic linker sequence which overcomes these potential problems. The *in vitro* expression of this feline flexi-IL-12 construct was demonstrated by Western blot analysis. Specific *in vitro* bioactivity was then shown using the dose dependent production of IFN γ from equine lymph node cells.

IL-18 is synthesised as a biologically inactive precursor molecule pro-IL-18, which is cleaved within the cell by the protease caspase-1 to produce bioactive mature-IL-18. This molecule then possesses the natural signalling peptide required for extracellular secretion. Inoculation of pro-IL-18 plasmid therefore must rely on the presence of endogenous caspase-1 for protein secretion. Immunisation with mature-IL-18 produces protein that lacks a signalling peptide and is inefficiently secreted from the cell. For these reasons, a construct was produced which encoded feline mature-IL-18 fused to a synthetic signal peptide IL-1 β receptor antagonist protein (ILRAP). ILRAP-IL-18 *in vitro* protein expression was analysed by Western blot analysis. It was found that unlike pro- and mature-IL-18 constructs, the ILRAP signal peptide facilitated secretion from the cell and further, was associated with higher relative expression than the previously used IL-18 expression construct. Secreted ILRAP-IL-18 was also shown to be bioactive by measuring the specific dose dependent production of human IFN γ from KG-1 cells. In addition, the feline IL-18 receptor was amplified from the total RNA of MYA-1 cells. Development of KG-1 cell lines expressing the feline IL-18 receptor should enable production of a more sensitive feline IL-18 bioassay.

In order to establish the *in vivo* activity of these new constructs, a vaccination trial was performed using an FeLV DNA vaccine. This vaccine consisted of two plasmids, one expressing *gag/pol* and the other expressing *envA* of FeLV and preliminary work confirmed their protein expression by Western blot analysis and fixed cell immunofluorescence. Apart from new plasmid constructs, two main modifications to the previous trial were made. Firstly, a more natural oronasal FeLV-A/Glasgow-1 viral challenge was used where previously the virus was administered into the peritoneum. Also, vaccine groups comprising the cytokines in combination and each alone were used in order to establish if protection from viraemia was due to IL-12 and IL-18 acting in synergy or whether the effect was due to either interleukin alone. It was found that the vaccine with ILRAP-IL-18 and the vaccine group with both cytokines elicited complete protection against FeLV viraemia. Feline flexi-IL-12 alone as an adjuvant was not found to be effective. The vaccine with ILRAP-IL-18 alone also produced significant protection against latent infection. This suggests that,

IL-18 was acting as an effective adjuvant to the FeLV DNA vaccine and that IL-12 was not an effective adjuvant and may even be producing an inhibitory effect.

Finally, four persistently viraemic animals from the control group were inoculated three times at weekly intervals with the DNA vaccine and ILRAP-IL-18. Periodic blood samples were taken in order to establish if this vaccine combination would provide any immunotherapeutic effect in terms of FeLV viral status and proviral load. The use of this vaccine demonstrated no beneficial effects on the parameters measured.

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DECLARATION

The work described in this thesis took place at the Department of Veterinary Pathology at The University of Glasgow Veterinary School. Practical work was carried out between October 1999 and October 2002. The author was personally responsible for all the practical work completed apart from where specifically mentioned. Haematology analysis in chapter 5 was performed by Ronnie Barron and Kenny Williamson in Veterinary Clinical Pathology and Histopathology, University of Glasgow. All FeLV blood screening in chapters 5 and 6 was performed by members of the Feline virus unit, University of Glasgow. FeLV p27 ELISA assays and virus neutralising antibody analysis was performed by Mike McDonald. Virus isolation experiments were performed by Mike McDonald, Dr Angela Pacitti and Joyce Simpson. Western blot analysis of non-neutralising antibodies and bone marrow culture was performed by Mathew Golder. The quantitative real-time PCR assay used in chapter 6 was designed by Stephen Dunham, Retrovirus Research Laboratories, University of Glasgow. The IL-12 bioassay was initially developed by Samantha Taylor, University of Glasgow and the IL-18 bioassay was developed by Linda McMonagle, University of Glasgow.

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CHAPTER 1: INTRODUCTION

1 INTRODUCTION

1.1 DNA vaccination

1.1.1 Introduction

The development of DNA vaccination originated from the discovery that injection of DNA expression vectors into mouse skeletal muscle generated the expression of protein without the requirement of a delivery system [Wolff *et al.* 1990]. Long-term persistence of injected plasmid DNA in mouse muscle was subsequently demonstrated by quantitative PCR [Wolff *et al.* 1992]. Soon after, it was found that plasmid DNA could be introduced into the skin of mice, by propelling DNA-coated gold microprojectiles directly into murine skin cells using a gene gun. An immune response against the expressed protein was also detected [Tang *et al.* 1992]. Subsequently, protection against challenge with lethal influenza virus was demonstrated by injection of chickens by intravenous (IV), intraperitoneal (IP) and subcutaneous (SC) injection with a haemagglutinin-expressing plasmid [Robinson *et al.* 1993]. Robinson *et al.* showed that two inoculations of 100µg DNA plasmid injected 4 weeks apart was sufficient to induce protection against viral challenge. This was the first demonstration that an antigen-expressing plasmid could be used as a DNA vaccine.

This first trial led to further investigation into this new concept in vaccinology. Inoculation via parenteral, mucosal and gene gun delivery induced protection against influenza virus in chickens and mice [Fynan *et al.* 1993]. Also, stimulation of strain-specific cytotoxic T lymphocytes (CTLs) to influenza A nucleoprotein DNA [Ulmer *et al.* 1993] and production of anti-human immunodeficiency virus (HIV) neutralising antibody [Wang *et al.* 1993] were demonstrated in trials in mice. This confirmed that both branches of the immune response may be stimulated by DNA vaccination.

This preliminary work has led to the development of DNA vaccines encoding antigens against a wide range of pathogens, including viruses, bacteria and parasites.

Additionally, plasmids encoding tumour antigens have also shown potential in tumour immunotherapy.

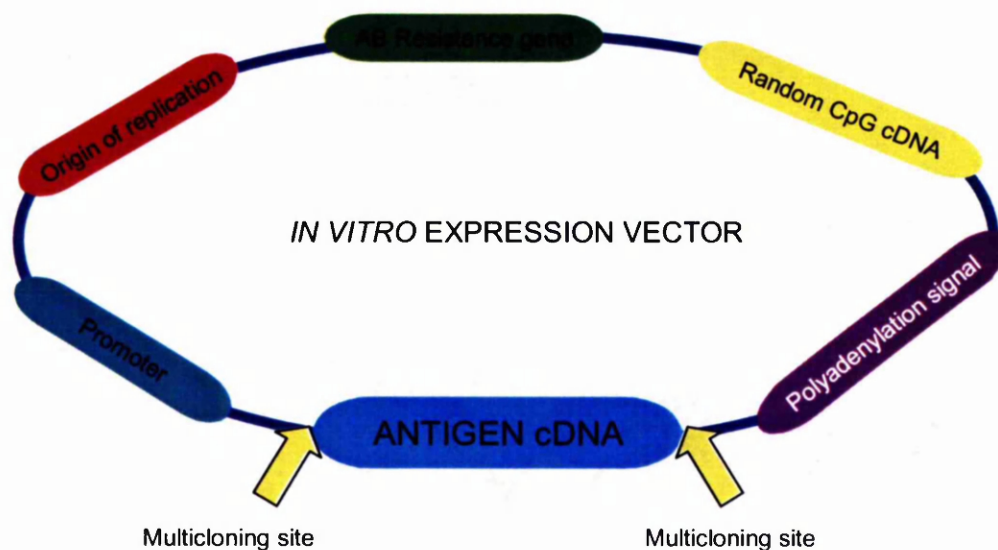
Up to date information on genetic immunisation can be found on the DNA vaccine website. The address is <http://www.dnavaccine.com/>

1.1.2 Design of DNA vaccine plasmid vectors

1.1.2.1 Basic plasmid design

The basic plasmid design for a DNA vaccine is shown in figure 1.1. A DNA vaccine consists of a plasmid that encodes an antigenic portion of a pathogen. The backbone of the plasmid is similar in structure to an *in vitro* expression vector. The basic elements that form this backbone may be split broadly into two groups. Firstly, replication and selection of the plasmid during bacterial culture dictates that a bacterial origin of replication and an antibiotic resistance gene are required. The second group consists of elements that assure efficient and maximal expression of antigen. These consist of a strong promoter suitable for expression in eukaryotic cells, a multicloning site for antigen cDNA insertion and a mammalian polyadenylation signal for efficient transcription termination. Also, the inclusion of CpG motifs has been shown to enhance immunogenicity which will be described later in the section.

Figure 1-1 Basic design of DNA vaccine plasmid



Promoter

Several promoters have been used to maximise the antigen expression of plasmid vectors. Reasonable expression has been shown using Simian virus 40 (SV40) [Moreau *et al.* 1981] and Rous sarcoma virus (RSV) [Gorman *et al.* 1982]. However, by far the highest gene expression has been demonstrated using the cytomegalovirus (CMV) immediate early gene promoter [Boshart *et al.* 1985]. The strength of this promoter was further enhanced by the inclusion of the first intron (intron A) of the immediate early gene [Chapman *et al.* 1991].

Polyadenylation signal

Two polyadenylation signals are used most frequently. These are the bovine growth hormone (BGH) and SV40 signals. A study has shown that BGH in combination with a CMV promoter and intron A elicited high expression of influenza A haemagglutinin and nucleoprotein antigenic protein. When used *in vivo* this plasmid protected mice from lethal challenge [Montgomery *et al.* 1993].

1.1.2.2 Further development of DNA vaccine design

Following the basic plasmid design described above, recent work has identified new features of the plasmid that are able to enhance or customise antigen expression still further.

Multiple gene expression

In certain cases it is necessary to simultaneously express two genes within the same plasmid. This has been made possible using bicistronic vectors. These vectors incorporate an internal ribosome entry site (IRES) between the two genes [Clarke *et al.* 1997]. Co expression of genes has also been investigated using the linear arrangement of separate promoter, gene and polyadenylation sites within the same vector [Iwasaki *et al.* 1997]. In this case it was found that co expression of B7-2 enhanced the epitope-specific CTL response to a non-immunogenic influenza nucleoprotein-encoding vector.

Directing antigen processing

In the cellular processing of an encoded protein, a limiting factor in the expression of MHC I complexes is the peptide concentration in the endoplasmic reticulum [Ortmann *et al.* 1994]. The generation of peptides for MHC I presentation takes place within the proteasome. Proteins can be targeted to the proteasome by a covalent link to the cellular protein ubiquitin. A study has shown that intracellular protein degradation of a lymphocytic choriomeningitis virus nucleoprotein is enhanced by fusion with the gene encoding ubiquitin. Injection of the DNA vaccine construct covalently linked to ubiquitin produced enhanced anti-viral CTL induction and protection, compared to nucleoprotein alone [Rodriguez *et al.* 1997].

Vaccines have also been designed where the antigen protein is directed towards sites of immune induction by fusion to certain ligands. In one example, two ligands were used, L-selectin and cytotoxic T-lymphocyte antigen. These are concerned with initiating entry to lymph nodes and binding to antigen presenting cells (APCs) respectively. These enhanced cellular immune responses in mice and by directing antigens to APCs, hastened the appearance of an immune response [Boyle *et al.* 1998].

Plasmids containing cDNA libraries

The concept of DNA immunisation has been broadened by using cDNA library fragments [Barry *et al.* 1995]. A cDNA library preparation of *Mycoplasma pulmonis* was found to confer protection in a mouse challenge model. It was postulated that approximately 1ng of DNA plasmid was required to elicit an immune response by genetic immunisation in mice. Each inoculation used 1-4µg of DNA and libraries containing 10^3 - 10^4 members were used [Barry *et al.* 1995]. The advantage of this type of approach is that an immune response may be elicited to a variety of antigenic genes. It also affords a means of screening for antigens most effective in providing immune protection for a particular pathogen.

Cell specific promoters

The use of promoters that direct the synthesis of antigen protein in certain cells has been investigated [Xiang *et al.* 1997]. In this study, vectors were constructed expressing rabies virus glycoprotein under the control of MHC I and MHC II promoters. The MHC I promoter has broad tissue specificity and the antigen under control of this promoter developed an immune response in mice comparable to a vector using SV40 promoter. The MHC II promoter restricts antigen expression to cells that express MHC II molecules such as dendritic cells (DCs), macrophages and B cells. Use of this promoter resulted in a relatively weak immune response against the DNA vaccine concerned.

Cytokine enhancement of DNA vaccines

Cytokines have been fused to antigens and have been incorporated into multigene expressing vectors. Depending on the cytokine used, this has been used to enhance or bias the immune response to the antigen [Iwasaki *et al.* 1997]. Use of cytokines as adjuvants to DNA vaccines will be discussed in subsequent chapters.

Use of CpG motifs

Recent interest has been shown in the use of immunostimulatory DNA sequences which may be used to enhance the performance of DNA vaccines. This idea emerged from the report that bacterial DNA can activate NK cells and stimulate interferon (IFN) production and tumour regression in certain mouse models, whereas vertebrate DNA does not [Tokunaga *et al.* 1984]. Further work showed that in contrast to vertebrate DNA, bacterial DNA could also stimulate B cell proliferation and immunoglobulin secretion [Messina *et al.* 1991]. Cytosine-phosphate-guanine (CpG) dinucleotides appear in microbial DNA at the expected frequency of 1 in 16, but are suppressed in the DNA of vertebrates, being present four times less often than in a random mix of bases. Also, CpG dinucleotides of bacteria contain mostly unmethylated cytosine, whereas vertebrate CpG consists of mainly methylated cytosine. A study using microbial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induced proliferation of murine B cells and secretion of immunoglobulin [Krieg *et al.* 1995]. This effect was not seen in the

same oligonucleotides lacking CpG dinucleotides, or where the CpG contained methylated cytosine. B-cell activation was found to be optimal when the CpG dinucleotide was flanked by two 5' purines and two 3' pyrimidines and this pattern has been termed a "CpG motif."

This has led to the theory that the immune system has evolved a way of detecting bacterial DNA and eliciting an appropriate immune response. The precise immune mechanisms against CpG DNA have since been explored. CpG DNA significantly up-regulates the expression of MHC II, CD86 (on circulating monocytes and activated B cells) and IL-12 in mouse DCs [Jakob *et al.* 1998]. This produces a corresponding enhancement of B and T-cell stimulation against these motifs. CpG DNA activates several signalling pathways in B cells that amongst other effects, induces secretion of IL-6, IL-10 and immunoglobulin [Yi *et al.* 1996; Redford *et al.* 1998]. Also, CpG DNA stimulates the maturation and activation of DCs and the conversion of immature DCs into professional antigen presenting cells (APCs) [Sparwasser *et al.* 1998]. Similar effects have been demonstrated on monocytes, macrophages and NK cells. The CpG motif induces lytic activity of NK cells in mice both *in vivo* and *in vitro* and stimulates an interferon gamma (IFN γ) response [Ballas *et al.* 1996; Cowdery *et al.* 1996]. The induction of lysis was found to be T and B cell independent and initiated indirectly by the secretion of IL-12, IFN α and β , and tumour necrosis factor alpha (TNF α). More recently, a CpG pattern recognition receptor, toll-like receptor-9 (TLR9) on DCs and other haematopoietic cells has been identified [Hemmi *et al.* 2000]. In this study, cells deficient in TLR9 were found to be unresponsive to CpG-containing DNA.

Having established that CpG DNA not only stimulates an immune response but biases the reaction towards Th1 type immunity, these sequences have been found to be potentially beneficial in improving DNA vaccine efficacy. This was initially confirmed intradermally in mice using a kanamycin based β -galactosidase vector. Vectors containing CpG sequences induced a stronger antibody response than vectors deficient of CpG DNA [Sato *et al.* 1996]. The CpG containing vector was shown to transcribe large amounts of IFN α , β , and IL-12 *in vitro*. Additionally, CpG sequence suppressed IgE synthesis and promoted IgG, IFN γ and IL-18 production [Roman *et*

al. 1997]. Subsequently, CpG DNA has been shown to induce resistance to *Listeria monocytogenes* infection in BALB/c mice [Krieg *et al.* 1998]. The potential potency of CpG DNA administered alone or in a DNA vaccine has also been demonstrated. Davis *et al.* showed that in mice, CpG oligodeoxynucleotides enhanced by five-fold the antibody response to a recombinant hepatitis B vaccine compared with a conventional adjuvant aluminium hydroxide [Davis *et al.* 1998].

The use of CpG motifs has been studied in various other species including primates. CpG DNA was used in combination with a commercial vaccine in an outbreak of hepatitis B in orangutans. Orangutans are hypo-responsive to this vaccine, but addition of CpG oligodeoxynucleotides greatly enhanced the seroconversion rate and antibody titres produced [Davis *et al.* 2000]. Also, cynomolgus monkeys intramuscularly injected with an HIV/SIV (SHIV) *tat*-expressing vector containing CpG sequences were protected against challenge with a highly pathogenic strain of the virus. Interestingly, monkeys injected with a CpG containing vector alone were partially protected, showing positive virus isolation but negative virus replication [Cafaro *et al.* 2001].

Preliminary feline and canine studies using CpG have also demonstrated enhanced immune responses. Peripheral blood mononuclear cells (PBMCs), lymph node cells and spleen cells were taken from dogs and cats and exposed to CpG sequences. Lymphocyte proliferation was observed in both lymph node and spleen cells but not in PBMCs [Wernette *et al.* 2002]. Also, the use of a CpG sequence in a minimalistic immunogenic defined gene expression vector (MIDGE) containing gp140 of FIV significantly reduced viraemia in cats compared to the use of a gp140 vector alone. This response corresponded with CTL activity as well as enhanced IL-12, IFN γ and IL-10 from PBMCs taken from these cats [Leutenegger *et al.* 2000].

1.1.3 Delivery of DNA vaccines

1.1.3.1 Route of administration

A DNA vaccine may be administered in several ways and the route of delivery can have an important influence on the level and the type of immune response elicited. The three most common routes of administration are intramuscular injection, intraepidermal delivery via a gene gun, and intradermal injection. Mucosal delivery via nasal, lingual, oral or vaginal routes has also been investigated as well as intrasplenic [Gerloni *et al.* 1997] and intrahepatic immunisation [Wolff *et al.* 1997].

Needle injection and gene gun

Vaccines designed for hypodermic inoculation of muscle or skin are usually suspended in saline or a saline mix containing a facilitator such as the anaesthetic bupivacaine, designed to increase uptake of DNA. Davis *et al.* showed that injection of DNA vaccine into muscle pre-treated with toxins or bupivacaine stimulated a 10-fold increase in antigen-expressing muscle cells and an enhanced immune response [Davis *et al.* 1995]. This mode of administration induced long-lasting immune responses without the requirement of booster injections [Davis *et al.* 1996]. It is thought this may be because mature muscle fibres are post-mitotic, resulting in long-term persistence and expression of episomally situated DNA.

Gene gun inoculation is most commonly administered intradermally, although the intramuscular route has also been used. The gene gun uses plasmid-coated gold particles, which on penetration of dermal cells allow solubilisation of plasmid DNA. This method of delivery transfects 20% of cells in the target area of the vaccine [Williams *et al.* 1991] and tissue stress resulting from inoculation possibly contributes to activation of DCs. It has been consistently shown that in comparison to needle injection, gene gun immunisation is more efficient, eliciting a comparable immune response with 100 to 5000-fold less DNA [Pertmer *et al.* 1995]. However, there is no empirical evidence to suggest that gene gun-based vaccination produces longer-term responses or superior protection against challenge than needle injection, even when larger doses of DNA are used [Barry *et al.* 1997].

In terms of the practical application of these two routes of administration, needle injection provides a cheap, simple and effective way of introducing the vaccine into the host. However, as previously mentioned, much larger amounts of DNA are required in order to induce a satisfactory level of immune response. This may be because the needle introduces DNA into extracellular spaces exposing it to nucleases from the interstitial fluid. Alternatively, the increase in hydrostatic pressure in the muscle from intramuscular inoculation may result in DNA being driven out of protein-producing cells decreasing protein expression.

Conversely, the gene gun route is itself limited by the small amount of DNA that can be introduced per inoculation. Administration of any more than 2.5µg at once can produce clumping of vaccine microparticles, which increases damage of the target tissue. Also the gene gun itself is expensive and requires inoculation into hairless skin that can lead to problems, especially in terms of veterinary use. These issues are being addressed currently by development of new generation guns.

Profound differences in the nature of immune responses elicited by gene gun and needle injection have been observed. Intramuscular injection tends to produce a mainly Th1 type immune response, with a higher IgG2a:IgG1 ratio, increased IFNγ production and low IL-4 production. This route of delivery therefore biases the immune system towards a cell-mediated response. Gene gun inoculation however tends to stimulate a Th2 orientated response and therefore humoral immunity, producing higher quantities of IgG1, less IFNγ and more IL-4 [Pertmer *et al.* 1996]. The gene gun can elicit a more Th1 type response with the co administration of cytokine genes such as IL-2, IL-7 and IL-12 [Prayaga *et al.* 1997]. In the case of intradermal injection, both Th1 and Th2 immune profiles have been reported [Pertmer *et al.* 1996; Raz *et al.* 1996].

A possible reason for this variation may be due to the amounts of DNA used in each case. Barry *et al.* 1997 demonstrated that when equal small amounts of DNA are used for both routes of administration, a predominantly Th2 response is elicited in both cases. When equal larger amounts were used as in intramuscular injection, the response shifted towards a Th1 profile in both cases. However other studies conflict

with this observation, showing that even when 1-2 µg DNA was injected into muscle mostly IgG2a antibodies were induced, whilst the same quantity of DNA stimulated IgG1 antibodies when delivered using a gene gun [Feltquate *et al.* 1997].

Another explanation for these differences may lie in the way the DNA is delivered to host cells. Injection of vaccine tends to deliver plasmid DNA to extracellular spaces where it is taken up by a mechanism that has not been fully elucidated. Gene gun inoculation however delivers DNA directly into cells. This difference may alter the processing of the antigen, leading to induction of different antibody responses.

Needle free jet

A new inoculation technique using the needle free jet device called Biojector™ has been used to administer DNA vaccines. It uses a CO₂ cartridge to propel the vaccine subcutaneously or intradermally. When used in rabbits to deliver a malaria vaccine, the biojector elicited 10-50 fold higher antibody titres and an improved ability to prime the immune system than using intramuscular needle injection [Aguiar *et al.* 2002].

Mucosal immunisation

There has also been recent interest in the delivery of DNA vaccines to the mucosal tissues. The earliest study was by Fynan *et al.* [1993], which showed that 2 intranasal inoculations of plasmid encoding influenza haemagglutinin protein stimulated IgG antibodies and protected 76% of mice against challenge. Further studies have demonstrated that mucosal vaccination tends to elicit a high IgG1:IgG2a ratio and Th2 associated cytokines, eliciting a Th2 orientated response [Kuklin *et al.* 1997]. The development of immune responses at distant mucosal sites as well as systemic immunity may be particularly useful in the defence against pathogens such as HIV, herpes simplex virus and influenza, which gain entry to the host via the mucosa.

1.1.3.2 DNA vaccine delivery systems

Lipopromoters

Incorporation of DNA vaccines into microparticles consisting of the biodegradable polymer poly(lactide-co-glycolide), allows the release of entrapped vaccine over extended periods of time and allows direct delivery of antigen into phagocytic APCs. This system was used by both intraperitoneal and mucosal delivery and successfully elicited IgG and IgM with the addition of IgA when administered to the mucosa [Jones *et al.* 1997].

A similar concept has combined DNA vaccines with cationic liposomes to form lipoplexes. Again this allows direct access to APCs by endocytosis, slow release of vaccine locally and protection from nuclease degradation. Intramuscular injection of lipoplexes containing DNA encoding hepatitis B surface antigen elicited stronger humoral and cellular immune responses than similar amounts of naked DNA [Perrie *et al.* 2001].

Live vectors

Live vector vaccines can be an effective way of delivering DNA plasmids encoding antigenic proteins to cells. These consist of attenuated strains of bacteria or viruses, which act as an inherent adjuvant to the vaccine due to their high immunogenicity. Specific antigenic proteins are encoded within the genome of the vector and once delivered to the host, vectors undergo their normal life cycle. Upon host expression, the encoded antigenic proteins are also transcribed, translated and become exposed to the immune system. Studies have shown that generally this delivery system is poor at stimulating antibodies to the pathogen, but due to limited replication at target sites of immunisation, they can be effective at inducing CD8⁺ T cell responses. Vectors that have commonly been used include strains of *Salmonella* [Pasetti *et al.* 2000], *Shigella* [Shata *et al.* 2001], and *E. coli* [Shiau *et al.* 2001].

1.1.4 Safety issues concerning DNA vaccination

Following the promising results produced by DNA vaccination, clinical trials have been conducted on healthy volunteers to assess the clinical safety and efficacy of plasmid DNA. There are several areas of safety that have caused concern.

1.1.4.1 Potential for integration

One of the main areas of concern is the possibility of integration of plasmid DNA into the host genome. Integration of plasmid DNA can result in three possible outcomes. Firstly insertion of DNA may have no effect on the host. If it produces disruption of a cellular gene, then this could potentially be mutagenic. Finally integration could inactivate a gene regulating cell division or activate an oncogene and the effect could, via a multistep process, be potentially carcinogenic [Amariglio *et al.* 1993].

DNA may be inserted into the host genome either by random or by homologous recombination. Homologous recombination is most likely to occur where there is simultaneous replication of both plasmid and host cells, and where homology exists between plasmid DNA and host DNA. In intramuscular DNA vaccination, the cells which process the plasmid are mainly non-dividing (myocytes and macrophages) [Mauro *et al.* 1969], the plasmids have very low sequence homology with mammalian DNA and plasmids do not contain a eukaryotic origin of replication. Therefore in theory, the risk of this type of integration is low [Donnelly *et al.* 1997].

Recent studies have addressed the persistence and distribution of plasmid DNA on intramuscular injection. After a single inoculation of a DNA malaria vaccine in mice, 3-30 copies of plasmid were detected per 10^5 muscle cells at up to 60 days post injection [Martin *et al.* 1999]. This study was unable to distinguish whether the plasmid was covalently linked to genomic DNA or merely adventitiously associated with it. However, assuming covalent linkage and that every integration resulted in mutation, this rate of mutation would be 3000-fold less than the spontaneous rate for mammalian genomes. However, the validity of this comparison is questionable, as spontaneous mutation of a single base would be less deleterious than integration of

large inserts of DNA. In addition, this study failed to monitor insertion of shorter DNA fragments, which may also give rise to mutation [Smith and Klinman, 2001].

A study of the tissue distribution of a DNA vaccine detected plasmid DNA in all highly vascularised tissues for 2 days after intramuscular injection. After this time, plasmid could only be retrieved from the muscle at the site of injection, where it persisted for up to 8 weeks [Parker *et al.* 1999]. Plasmid was detected initially in the gonads, giving rise to the risk of heritable defects should integration take place. To date however, no further work has been published in this area. It should be noted that during these studies, no adverse effects were detected in any of the clinical chemistry, haematology or histopathology of these animals.

In conclusion, evidence suggests that of the vaccines tested, integration is a rarity. To date, there is no proof that DNA vaccines can be carcinogenic or produce phenotypic mutation.

1.1.4.2 Autoimmunity

Another potential danger of DNA vaccination is the development of an immune response to host DNA. Studies have shown that the proportion of myotubes transfected with plasmid DNA is only 1-5% [Wolff *et al.* 1990]. Therefore it is unlikely that an immune response against these cells would be clinically significant.

Of the studies performed in which autoimmunity has been elicited, this has failed to produce autoimmune disease. Repeated immunisation of mice with plasmid DNA brought about a 3 to 4-fold rise in B cells secreting anti-mammalian IgG antibodies. However, no disease was demonstrated in either normal mice or those prone to systemic lupus erythematosus [Klinman *et al.* 1997; 2000].

1.1.4.3 Tolerance

It is theoretically possible that DNA vaccination could induce host tolerance to antigen rather than protective immunity. Generally, the amount of antigenic protein synthesised is estimated to be small and this protein often persists for long periods of

time, which makes immunotolerance a genuine concern. Studies have shown evidence of neonatal tolerance in mice immunised with a DNA vaccine for malaria. Mice immunised at 2-5 days of age failed to mount antibody, cytokine or cytotoxic responses on rechallenge with the vaccine [Mor *et al.* 1996]. This tolerance was found to persist for greater than a year and also interfered with immune responses to subsequent challenge. Susceptibility to tolerance was found to increase with higher doses and was not MHC restricted [Ichino *et al.* 1999].

In contrast, there is much work that demonstrates protective responses in neonates. A plasmid encoding lymphocytic choriomeningitis virus cDNA was found to induce long-term CD8⁺ and humoral responses when injected within hours or days of birth [Hassett *et al.* 2000]. Also, neonatal mice injected with plasmids expressing influenza antigens were protected against lethal challenge whereas those injected with inactivated virus were not [Bot *et al.* 1998].

In conclusion, it is clear that the development of tolerance to DNA vaccination requires further investigation, particularly in individuals that are most likely to be unresponsive such as the young.

1.1.4.4 CpG effect

In terms of safety, the major concern with CpG DNA is its ability to bias the cytokine response of the host towards a Th1 profile. This could increase the risk of Th1-mediated organ-specific autoimmune diseases such as allergic encephalomyelitis or could increase susceptibility to infection requiring a strong Th2 response. There have been differing reports on this issue. Klinman *et al.* have shown that repeated administration of CpG DNA at least twice a month produced no adverse effects in mice [Klinman *et al.* 1999]. However CpG DNA stimulated quiescent myelin basic protein-specific T cells to become effector cells able to transfer experimental allergic encephalomyelitis [Segal *et al.* 1997]. Again, evidence suggests that the risk of CpG DNA requires further investigation before concerns about safety can be fully answered.

1.1.4.5 Development of anti-DNA antibodies

The stimulation of antibodies against plasmid DNA itself is a potential problem, as this could theoretically contribute to the onset of certain autoimmune diseases such as systemic lupus erythematosus [Klinman *et al.* 1991]. Other important factors that predispose to this syndrome are genetic susceptibility and immune dysfunction.

It is, however, relatively difficult to induce antibodies to purified double stranded DNA (dsDNA). The DNA must first be denatured, complexed with methylated bovine serum albumin and co administered with complete Freund's adjuvant in order to stimulate this kind of response [Gilkeson *et al.* 1989]. Further studies analysed the immunisation of mice with complexes of *E. coli* dsDNA prepared in this way. Both normal and lupus-prone mice developed anti-dsDNA antibodies [Gilkeson *et al.* 1995]. However, survival of these mice was actually increased compared to the controls, with immunised mice displaying less clinical expression of the disease [Gilkeson *et al.* 1996]. Several studies using non-denatured, purified dsDNA as vaccines have failed to detect anti-DNA antibodies [Jiao *et al.* 1992]. One study detected a 35-60% rise in anti-DNA titres on DNA vaccination of normal and lupus-prone mice, but this had no effect on either the onset or the severity of autoimmune disease [Klinman *et al.* 1997].

1.1.5 Mechanism of DNA vaccination

The process by which DNA vaccines exert their effect on the immune system has not yet been fully elucidated. Evidence gathered so far has led to theories regarding the probable processing pathways from host cell entry to immune cell activation.

1.1.5.1 Entry of DNA vaccine into host cell

Once administered to the host, plasmid DNA must gain access to host cells in order to produce expression of antigen. In contrast to viruses, naked DNA lacks the structural elements necessary for entry into cells. The precise mechanism of cell entry remains unclear although it has been shown that cells can spontaneously take up nucleic acids. For example, myocytes have been shown to take up DNA, and initiate transcription

and translation into protein [Davis *et al.* 1993]. It has been shown that DNA is internalised by muscle cells near the injection site within five minutes by muscle cells, with rapid dispersion through the muscle over the following few hours [Dupuis *et al.* 2001]. Attempts have been made to enhance this uptake by the addition of cationic lipids that are able to bind DNA and facilitate transport across the cell membrane [Felgner *et al.* 1987]. Alternatively, DNA may be administered directly into the cell by use of a gene gun [Tang *et al.* 1992].

1.1.5.2 Antigen expression and uptake

Plasmid DNA is maintained episomally within host cells and the DNA is transcribed and translated into protein using the components of the cell. This results in production of vaccine-encoded antigenic protein, which may then be exposed to the immune system. Stimulation of either humoral or cytotoxic immune responses require presentation of antigen protein to MHC class I or class II molecules respectively. The mechanism by which this occurs from intramuscular injection has led to three possible pathways which have been reviewed by Leitner *et al.* [2000a], and are summarised in figure 1.2.

Antigen is presented directly by transfected myocytes

It has been suggested that myocytes themselves may present antigen and elicit T cell responses directly. Evidence to support this demonstrates that T cells are able to recognise myocytes in certain myopathies [Hohlfeld *et al.* 1993] and also in response to DNA vaccination. Yokoyama *et al.* demonstrated an antigen-specific local inflammatory response at the intramuscular injection site of a vaccine [Yokoyama *et al.* 1997]. Also a study has shown that where influenza nucleoprotein (NP) antigen production is restricted to C2C12 myoblasts, this is sufficient to induce a CTL response in C3H mice [Ulmer *et al.* 1996].

However it is the general view that in order to induce cytotoxic T-lymphocytes (CTLs), a cell requires at least two signals, an MHC I peptide complex and a co stimulatory molecule such as CD80 (B7.1) and CD86 (B7.2). Since expression of

both these molecules on myocytes is low, it is unlikely that muscle cells directly induce T cell responses.

Antigen is transfected into and presented by specialised bone marrow-derived antigen presenting cells (APCs)

A more likely mechanism of antigen presentation is through DCs, which are able to function as APCs. These cells are known to express both MHC I and II, as well as co-stimulatory molecules such as B7.1 and B7.2. This makes them highly efficient at presenting antigen to T lymphocytes. Various studies using chimeras have shown that CTL induction is restricted to MHC molecules of the bone marrow rather than the myocytes of the host. This was the case for both intramuscular and gene gun vaccination [Corr *et al.* 1996; Doe *et al.* 1996; Iwasaki *et al.* 1997]. It has therefore been suggested that a small number of APCs may be transfected with plasmid DNA at the site of injection. In the case of intramuscular injection however, the relative scarcity of DCs and macrophages in muscle make this unlikely.

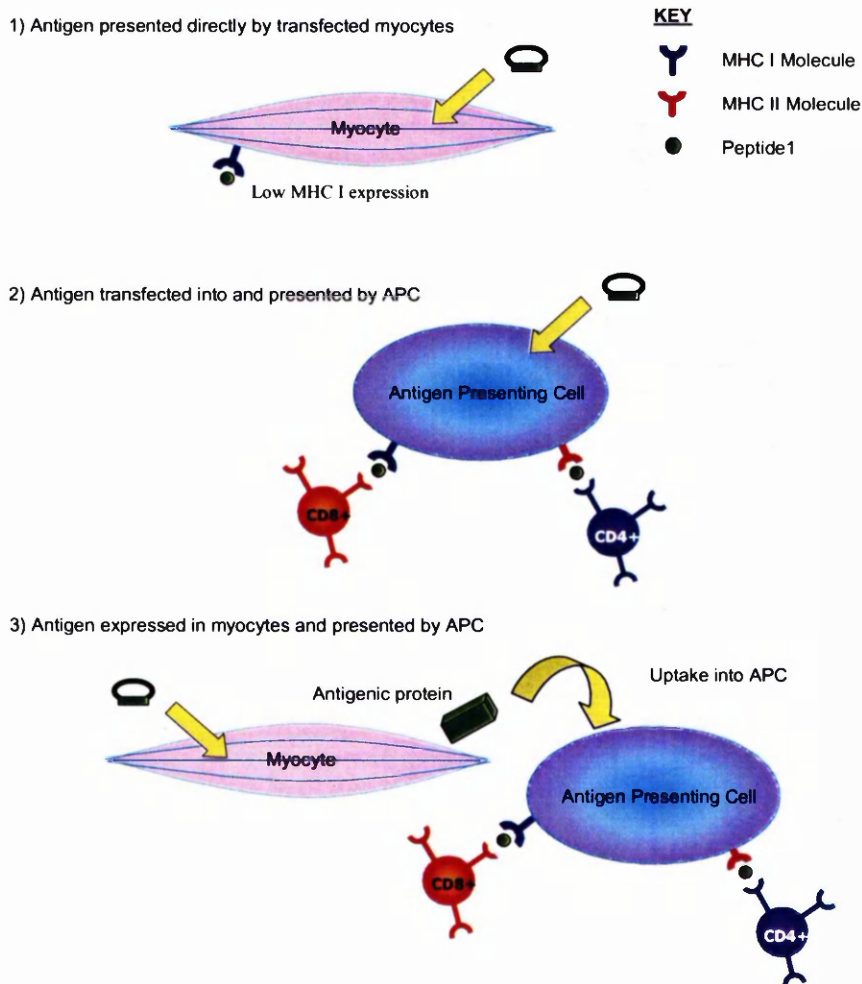
In intradermal inoculation, this hypothesis is more feasible due to the abundance of APCs present in the dermis. Introduction of plasmid DNA into the skin was shown to result in discrete foci of epidermal and dermal cells. These included cells with dendritic morphology, which were found to contain the injected antigen [Raz *et al.* 1994]. In fact Raz *et al.* have suggested that the true effector cells involved in intramuscular injection may actually be within the skin through which the needle has passed.

Gene gun immunisation has been shown to directly transfect DCs that were recovered from lymph nodes draining the inoculation site. These cells were specifically identified using antibodies to an intact surface protein encoded by co transfected DNA [Porgador *et al.* 1998]. This has also been confirmed by demonstrating the presence of DNA-coated gold particles in the cytoplasm of DCs after gene gun inoculation [Condon *et al.* 1996].

Antigen is expressed in myocytes and presented by APCs ("cross priming")

A hypothesis that has gained popularity is that "cross priming" of APCs occur, leading to antigen presentation. In this process, it is thought that the APC is exposed to antigen manufactured by myocytes and acts as a "mediator cell" allowing antigen presentation to T lymphocytes. However, the way in which APCs apprehend the antigenic protein is still in debate. There are three possibilities. Firstly, the DC may be directly transfected with plasmid DNA as in the case of gene gun immunisation described previously [Porgador *et al.* 1998]. Alternatively, soluble antigen secreted by myocytes may be taken up from interstitial spaces surrounding the APC. Finally, APCs may engulf cells that have been injured or killed by the vaccine or its function

Figure .1-2 Antigen expression of DNA vaccine plasmid



There has been much interest recently in the possible role of apoptotic cell death in DNA vaccine efficacy, so this will be explained in more detail.

1.1.5.3 Signalling and activation of dendritic cells

It has been suggested that DC activation is dependent on recognition and binding to certain cellular signals. It has been found in a gene gun immunisation trial, that priming of specific CTLs is dependent on the co expression of antigen encoding DNA and the expression of B7.2 antigen [Porgador *et al.* 1998]. Also a study has shown that DCs recognise antigen from cells undergoing apoptosis and subsequently induce class I-restricted CTLs [Albert *et al.* 1998]. Together, these ideas have led to the idea that cell death induced by transfection of host cells becomes a signal for activation of DCs as they supply the necessary "danger signals" [Matzinger *et al.* 1998]. This cell death of antigen-expressing myocytes on intramuscular injection of DNA depends on MHC II restricted CD4⁺ activation of T cells, not MHC I or perforin-mediated lysis, and seems to be antibody mediated [Payette *et al.* 2001].

Destruction of transfected cells tends to be restricted to those that harbour high copy numbers of plasmid [Restifo *et al.* 2000]. This has been demonstrated by studies of plasmid DNA vectors containing replicons which, when transfected into cells launch a self-replicating cycle of the plasmid-encoded antigen. This vector was compared with a traditional DNA plasmid and was found to induce the same immune response and protection against tumour antigen with 100 to 1000-fold less replicon DNA. Neither vaccine produced higher antigen levels than the other; instead the effect was associated with caspase-dependent apoptotic death of the host cells. This death facilitated antigen uptake by DCs, potentially enhancing the immunogenicity of the vaccine [Ying *et al.* 1999; Leitner *et al.* 2000b]. A theory has been put forward that low efficacy of conventional DNA vaccines may be due to a lack of cell death, preventing exposure of DCs to the "danger signals" required for activation.

Sasaki *et al.* have deliberately used apoptotic death to enhance a DNA vaccine to influenza virus. They immunised mice with the vaccine and vectors expressing partially inactivated caspase-2 or the chimeric version of caspase-2 grafted onto caspase-3. These enzymes mediate programmed cell death and mutated versions were

used in order to allow antigen expression before apoptosis. They showed that the apoptotic bodies produced were engulfed by APCs and claim that the increased T cell response was the strongest vaccine adjuvant effect shown to date [Sasaki *et al.* 2001].

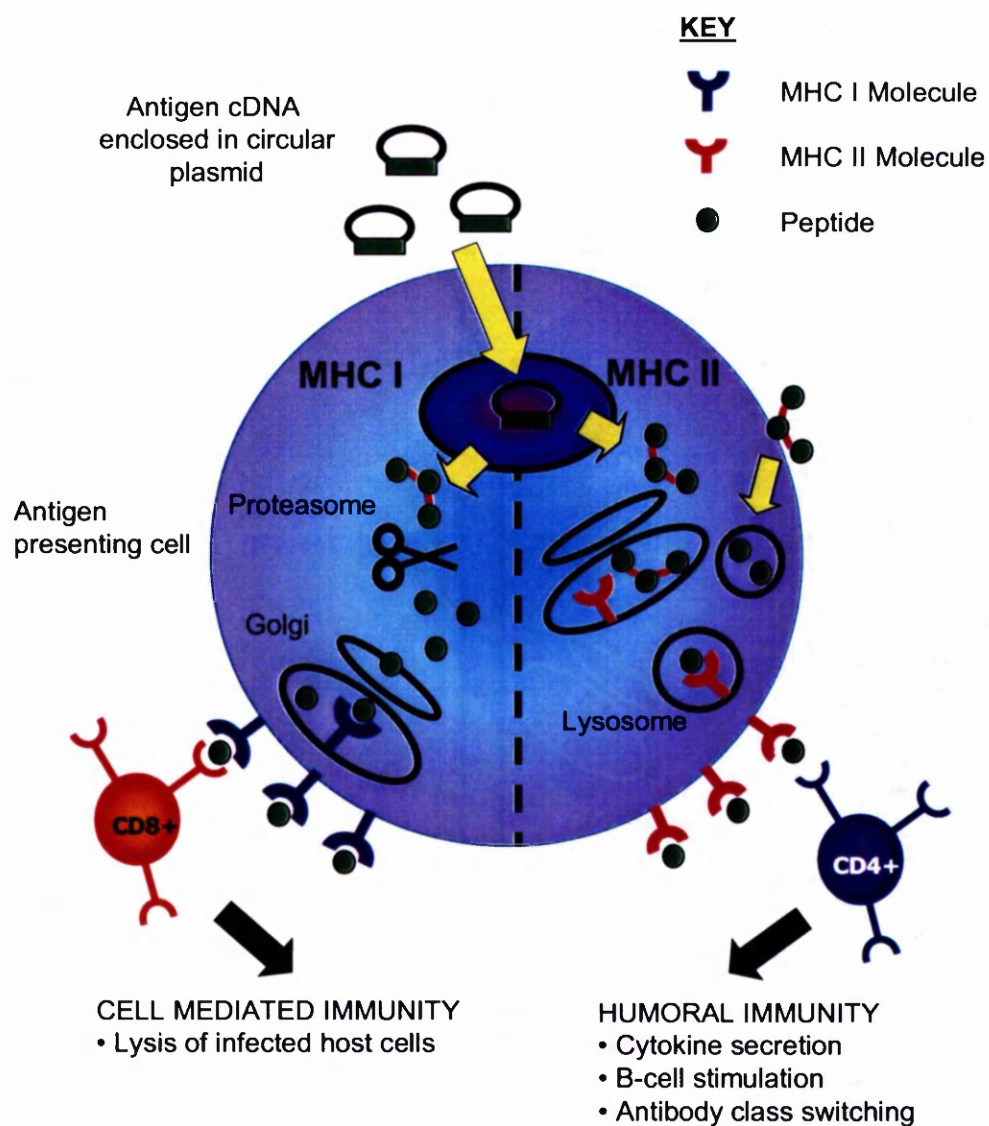
In conclusion, it is generally accepted that APCs provide the link between inoculation of antigen encoding DNA and exposure of antigenic protein to the cells of the immune system. Whether this DNA is transfected directly into the APC and the protein synthesised, or whether the APC takes up protein synthesised elsewhere is unclear. Some studies have shown that a combination of these two mechanisms take place. For example on intradermal injection of a DNA vaccine, APCs were shown to process extracellular proteins, as well as directly transfected plasmid [Corr *et al.* 1999]. In addition, as mentioned in 1.1.2.2, fusion of antigenic cDNA to cytotoxic T-lymphocyte antigen encouraged binding of antigenic protein to APCs which was found to hasten and enhance the immune response elicited compared to unlinked vaccines [Boyle *et al.* 1998]. Whichever pathways occur however, antigens must then be processed and presented to immune cells to stimulate a protective immune response.

1.1.5.4 Presentation of antigen within the APC

Once exposed to environmental antigens, the APCs migrate to the draining lymph nodes where antigen presentation takes place. During this time, the APC undergoes a functional transition from antigen-uptake to presentation of T-cell-stimulatory signals through MHC I and MHC II complexes. DCs have been shown to have superior antigen-presenting capacity compared to other APCs during DNA vaccination. A possible explanation for this has been the discovery of a DC-specific surface receptor called DC-SIGN (DC-specific ICAM-3 grabbing non-integrin) [Geijtenbeck *et al.* 2000]. These authors suggest that DC-SIGN interacts with ICAM-3 (CD50) on resting T-cells, which establishes initial contact with the T-cell and allows the T-cell receptor to scan the surface of the DC for specific MHC complexes. This may be important in the adaptive immune response to DNA immunisation, where there may be very few antigen-presenting DCs available.

Antigens encoded within DNA vaccines have the capability to induce both cell mediated and humoral immunity depending on factors such as the antigen concerned and the mode of administration. These antigens are therefore able to enter both the MHC I and MHC II processing pathways of the APC (figure 1.3).

Figure 1-3 Antigen presentation via the MHC I and MHC II pathways



Antigenic protein entering the MHC II pathway may be endogenous within the APC [Oxenius *et al.* 1997] or taken up from the extracellular environment. It enters lysosomes where it is proteolysed to peptides, which then bind to MHC II molecules. These complexes are displayed on the cell surface where they are recognised by CD4⁺ T-helper cells [Male *et al.* 1996a]. T-helper cells are divided into 2 groups. Th1 cells

secrete $\text{IFN}\gamma$, $\text{TNF}\alpha$ and IL-12, thereby promoting cellular immune responses. Th2 cells secrete cytokines such as IL-4, IL-5 and IL-13, which stimulate the maturation of humoral responses. CD4^+ T cells act on mature and naive B cells, stimulating growth and clonal expansion when the cells come into contact with antigen. T-helper cells may also bring about class switching which involves changing the class of antibody produced by the cell, from for example IgM to IgG. The precise role of CD4^+ cells in the generation of CD8^+ cytotoxic T-lymphocytes is at present unclear. However studies have shown that mice that lack CD4^+ cells show relatively normal CD8^+ CTL responses but impaired CTL memory. As a consequence of this, vaccination of CD4^+ deficient mice is significantly less effective [Von Herrath *et al.* 1996].

The origin of antigenic protein processed via the MHC I pathway is yet to be fully determined. Some believe the protein must be synthesised endogenously, others that "cross priming" occurs as explained above. Once inside the APC, antigenic protein is degraded to peptides within the proteasome. These peptides are transported to the endoplasmic reticulum by TAP transporters where they bind to MHC class I molecules and $\beta 2$ -microglobulin. These complexes are then displayed on the cell surface where they may be recognised by cytotoxic CD8^+ T-cells [Male *et al.* 1996a]. These cells are able to lyse infected host cells which display antigen on their MHC I molecules, providing a crucial role in the spread of infection. There are various ways that lysis may take place. The cytotoxic T-cell may bind the target cell and release granules, which can non-specifically lyse the target cell in a Ca^{2+} dependent manner. In addition, Fas antigen, a 48kDa member of the TNF receptor family, has been shown to be important. Fas ligand present on the cytotoxic T-cell can bind Fas antigen on the target cells. This complex can then bring about apoptosis of the target cell [Male *et al.* 1996b].

1.1.6 DNA vaccination of domestic animals

The concept of DNA vaccination has been explored in various species. The strength and type of immune response elicited, has varied with parameters such as the vaccination protocol, the amount of vaccine used, route and timing of administration, type of antigen and species concerned. Studies investigating genetic immunisation in feline (table 1.1) and canine (table 1.2) systems have been summarised below.

As shown in table 1.1, most studies of feline retrovirus DNA vaccines *in vivo* have used intramuscular inoculation of plasmid vectors comprising one or more antigenic genes. These studies clearly show that the type of immune response elicited can vary according to many parameters. In the case of FIV, injection with gp120 cDNA induced a predominantly humoral response [Cuisinier *et al.* 1997], whereas inoculation with a defective mutant provirus induced antigen-specific CTLs and no humoral response [Hosie *et al.* 1998]. It could be concluded that the contrasting immune response may be due to the different FIV genes used, which stimulate different pathways in the immune system. However, when these two studies are compared, the immunisation protocol, dose of vaccine and strain of virus used for challenge were different. All of these parameters may have influenced the immune response elicited. As explained in 1.1.3, the way a DNA vaccine is administered can influence the response produced as much as the composition of the vaccine itself. This highlights the empirical way in which the optimal conditions for a particular vaccine are defined. An additional problem, particularly relevant in human studies, is the use of murine experimental systems to investigate vaccines for pathogens of other species. The response elicited by a vaccine in mice cannot necessarily be extrapolated to a different species, especially in the case of DNA vaccination where immunisation parameters are so empirical.

As detailed in table 1.1, certain cytokines have been used as adjuvants to DNA vaccines for feline retroviruses. IFN γ used as an adjuvant to an FIV DNA vaccine, increased the level of protection to viral challenge [Hosie *et al.* 1998] and IL-12 elicited a high degree of protection with a DNA vaccine to FIV delivered by gene gun [Leutenegger *et al.* 2000]. IL-18 was also shown to enhance FIV-specific CTL responses when used in combination with a DNA vaccine [Dunham *et al.* 2002]. The

efficacy of IL-12 and IL-18 as vaccine adjuvants are described in chapters 3 and 4 respectively. Some studies have shown that a DNA vaccine that is not protective alone can provide complete protection against challenge when accompanied by these adjuvants. Hanlon *et al.* demonstrated that IL-12 and IL-18 together with a DNA vaccine encoding FeLV-A *gag/pol* and *env* genes, produced complete protection from FeLV challenge which was not induced by vaccine alone [Hanlon *et al.* 2001]. The effect was found to be associated with an increase in antigen-specific CTLs [Flynn *et al.* 2000a]. This shows that IL-12 and IL-18 biased the immune response towards cell-mediated immunity, which allowed a more effective immune response to viral challenge. The work in this thesis further investigates the adjuvant role of these feline cytokines either alone or combination, to a FeLV DNA vaccine *in vivo*.

Table 1-1 Feline DNA vaccination studies

*FIPV = Feline infectious peritonitis virus

Pathogen	Route	Antigen cDNA	Delivery/ adjuvant	Immune response	Study
FeLV	IM	<i>gag/pol + envA</i>	Plasmid vector, IL-12 + IL-18	↑ virus-specific effector CTL, <i>gag/pol</i> recognition > <i>envA</i> No antiviral antibodies produced Significant protection in vaccine + IL-12 + IL-18	Hanlon <i>et al.</i> 2001; Flynn <i>et al.</i> 2000a
FIV	IM	FIV gp120 and p10	Plasmid vector	gp120 induced humoral response and some maintained low proviral load and controlled replication gp120 + p10: complete humoral response and transient drop in proviral load	Cuisinier <i>et al.</i> 1997
FIV	IM	<i>env</i> (wild-type + mutated gene)	Plasmid vector	Enhanced infection low anti- <i>env</i> antibodies	Richardson <i>et al.</i> 1997
FIV	IM	defective mutant provirus	Plasmid vector, IFN γ	Vaccine alone: CTL to FIV <i>gag</i> and <i>env</i> , ↓ viral loads, No antiviral antibodies Vacc + IFN γ : ↑ protection	Hosie <i>et al.</i> 1998
FIV	IM + Mucosal	Nucleocapsid	FIV DNA as adjuvant to gp120 protein	FIV DNA ↓ viral load and ↓ anti-FIV humoral response of protein vaccine	Cuisinier <i>et al.</i> 1999
FIV	IM	defective mutant provirus of FIV petaluma strain and Glasgow-8	Plasmid vector	Some protection of petaluma strain, no effect on more virulent Glasgow-8 strain	Hosie <i>et al.</i> 2000
FIV	IM	provirus with deletion in accessory gene <i>vif</i>	Plasmid vector	Protection with low CTL response, high anti- <i>env</i> antibodies, no anti- <i>gag</i> antibodies	Lockridge <i>et al.</i> 2000
FIV	Gene gun	FIV gp140	MIDGE vector, IL-12, IL-16, CpG motif	No protection with vaccine alone, high protection with either of 3 adjuvants. Weak CTL response, No FIV specific antibodies. Moderate cytokine response	Leutenegger <i>et al.</i> 2000
FIV	IM	replication defective provirus	Plasmid vector with CMV or 5'LTR promoters, IFN γ	Vaccine alone: significant protection Vaccine + IFN γ : highest virus-specific lysis with CMV promoter, highest protection with 5'LTR promoter	Flynn <i>et al.</i> 2000b
FIV	IM	replication defective Reverse Transcriptase and Integrase genes	Plasmid vector, IL-12 and IL-18	7/30 animals remained virus free. IL-18 cDNA : more consistent CTL response IL-12 cDNA: no enhancement of immune response	Dunham <i>et al.</i> 2002
Rabies virus	IM + ID	glycoprotein G	Plasmid vector	VNA level ID > IM	Osorio <i>et al.</i> 1999
FIPV*	2 x ID + 2 x IM	membrane and nucleocapsid	Plasmid vector, IL-12	No protection elicited by DNA vaccine IL-12 increased susceptibility of animals to virus	Glansbeek <i>et al.</i> 2002

Table.1-2 Canine DNA vaccination studies

Pathogen	Route	Antigen cDNA	Delivery/ adjuvant	Immune response	Study
canine distemper virus (CDV)	IM + gene gun	Haemagglutinin and fusion protein	Plasmid vector	IM: IgG2a antibody (Ab) gene gun: haemagglutinin produced IgG1. Fusion produce mixed Ab response. Both produced class I-CTL and protection from challenge	Sixt <i>et al.</i> 1998
CDV	IM	Nucleocapsid, fusion, and attachment protein	Plasmid vector	Positive virus neutralising antibodies and protection from challenge	Cherpillod <i>et al.</i> 2000
CDV	IM	Nucleocapsid of CDV	Plasmid vector	Positive specific IgG response, with no IgM antibody peak. Lower response than conventional protein vaccine	Griot-Wenk <i>et al.</i> 2001
Rabies virus	IM	PV strain glycoprotein	Plasmid vector	Positive virus neutralising antibodies and protection from challenge	Perrin <i>et al.</i> 2000
Rabies virus	IM + ID	glycoprotein G	Plasmid vector	VNA level IM > ID	Osorio <i>et al.</i> 1999
canine parvovirus	IM	VP1 gene	Plasmid vector	Increase in IgG titres and complete protection from virus	Jiang <i>et al.</i> 1998
canine oral papilloma virus	mucosa via Powerject device	L1 gene	particle mediated DNA delivery	Complete protection from challenge. Cell mediated lymphoproliferative and humoral responses observed	Stanley <i>et al.</i> 2001

1.2 Feline Leukaemia Virus

1.2.1 Introduction

Feline leukaemia virus (FeLV) was originally identified by William Jarrett in 1964 [Jarrett *et al.* 1964a; Jarrett *et al.* 1964b]. In this work, a cell-free extract was prepared from a naturally occurring lymphosarcoma in the mediastinum of a cat and was injected into four kittens within 12 hours of birth. These animals were then reared in isolation and subjected to frequent clinical examination. Within six months of injection, enlarged superficial lymph nodes could be detected and several cats had developed splenomegaly. Within eighteen months all of the cats had either died or had to be euthanased, having developed signs associated with leukaemia or lymphosarcoma. Post mortem examination confirmed that all cats demonstrated leukaemic disease based on gross and histologic diagnosis [Jarrett *et al.* 1964a].

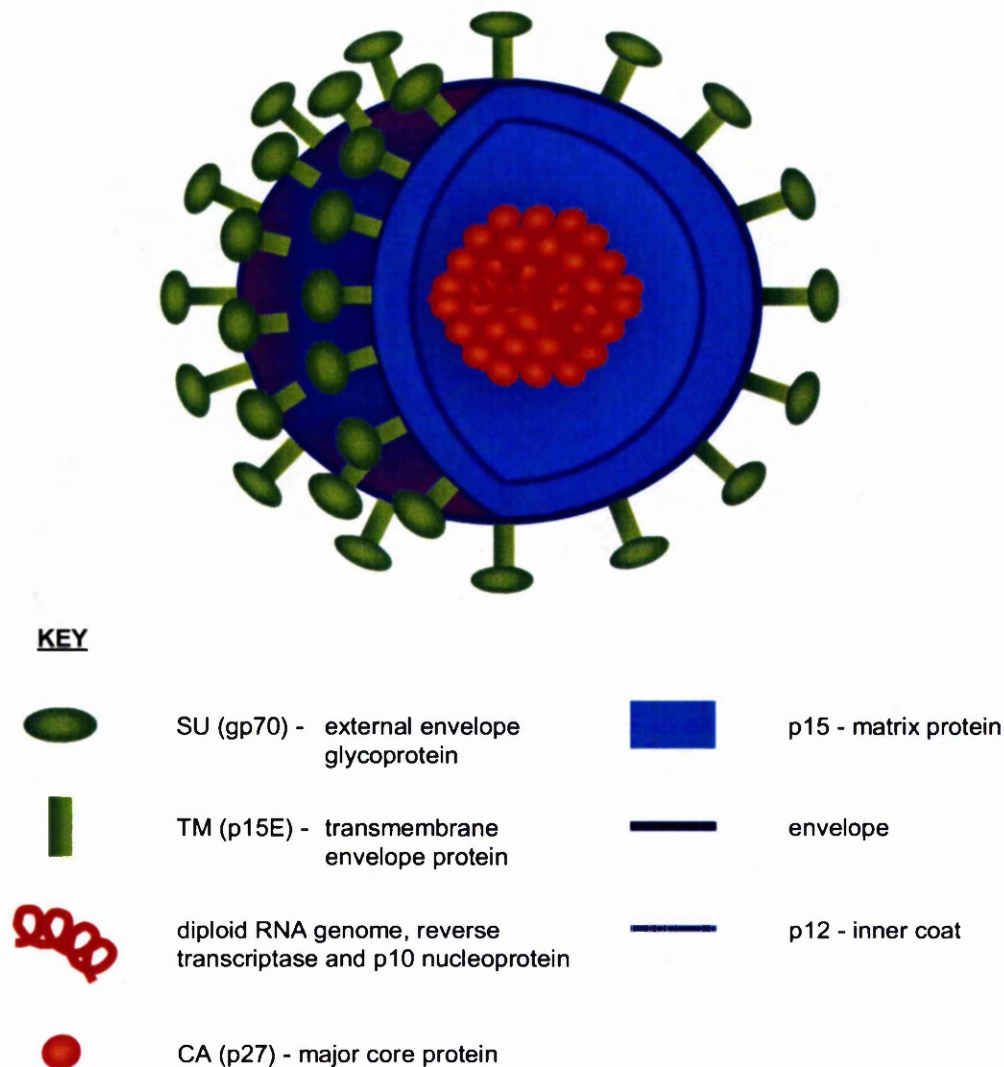
The hypothesis that the lymphosarcoma induced by the inoculation of the tumour extract contained some kind of infectious agent was tested by electron microscopic examination of the mass. The cells examined contained highly vesiculated areas and virus-like particles were identified within the vesicles and in the intercellular spaces. These particles comprised an electron-dense nucleoid enclosed in a densely stained double membrane. Large numbers of similar particles were detectable in short-term cell cultures of the tumour cells. This virus was designated "feline leukaemia" virus due to its strong structural similarity to murine leukaemia virus (MuLV) [Jarrett *et al.* 1964b].

The isolation of FeLV also led to the identification of feline sarcoma virus (FeSV) by Snyder and Theilen who found that naturally occurring fibrosarcoma could be reproduced by inoculating experimental cats, in a similar way to FeLV [Snyder and Theilen, 1969]. Further work demonstrated that FeSVs arise from recombination between FeLV and cellular proto-oncogenes, but are not transmitted from cat to cat in nature.

1.2.2 Structure of FeLV

FeLV is a spherical enveloped virus, 100nm in diameter (figure 1.4). The structure of the virus is reviewed by Granoff and Webster [1999], and Fields and Knipe [1990].

Figure 1-4 Diagram representing the structure of feline leukaemia virus



The FeLV viral genome comprises two linear 8.3kb positive-sense single stranded RNA molecules linked together to form a dimer. As shown in figure 1.5, the encoded gene order from 5' to 3' is *gag/pol/env* and as in cellular RNA, the genome has a 5' cap and 3' polyadenylation signal. Encoded in the viral RNA is reverse transcriptase, which on infection of the cell reverse transcribes the genome into DNA. This then becomes stably integrated into the host DNA to form a provirus.

The FeLV genome also contains U3, U5 and R sequences situated at the 5' and 3' ends of the genome. These are copied to form the proviral long terminal repeat (LTR) sequences and function in the initiation of transcription, polyadenylation of viral RNA and promotion of viral transcription. The 5' LTR acts to initiate transcription of the provirus, whereas the 3' LTR functions mainly as an RNA processing signal. On replication of the host cell, copies of the viral protein and DNA are manufactured by the machinery of the cell to form complete viral particles, which are then released.

Figure 1-5 Diagram of FeLV genome



The proteins p10, p15, p15E, p27 and gp70 produced from *gag*, *pol* and *env* of the FeLV genome were named according to their sizes in kilodaltons. However recently the nomenclature of these proteins has changed to NC, MA, TM, CA and SU respectively. The *gag* gene encodes polyprotein precursors of internal non-glycosylated core proteins. The major capsid protein (CA) forms the icosahedral core particle. The matrix protein (MA) forms an outer layer on the capsid and the nucleocapsid protein (NC) is associated with the virion RNA.

Proteins encoded by the *env* gene are required for the adsorption and entry of retroviral particles. These proteins are not necessary for virion assembly or budding as normal appearing virions can be made and released in their absence [Dickson *et al.* 1982]. The envelope of the virus is formed from the budding process as the virus leaves the host cell and consists of phospholipid bilayer derived from the plasma membrane. This envelope is studded with complexes of two *env*-coded proteins: surface glycoprotein (SU) and the transmembrane anchor protein (TM) which form trimers on the envelope surface [Hunter *et al.* 1990]. Variations in the SU protein separate FeLV into subgroups, FeLV-A, FeLV-B and FeLV-C [Sarma and Log, 1973; Russell and Jarrett, 1976] and FeLV-T [Rohn *et al.* 1998].

The virion enzymes protease, reverse transcriptase (RT) and integrase (IN) are encoded by the *pol* or polymerase gene of the genome. This arises from a *gag-pol* precursor, which is produced due to suppression of the *gag* termination codon.

1.2.3 Replication of FeLV

All retroviruses follow a similar replication cycle which is summarised in a diagram at the end of this section (figure 1.6).

1.2.3.1 Attachment of the virus to a specific cell-surface receptor

The initiation of replication takes place by the binding of the SU protein on the viral envelope to a specific receptor found on the host cell surface. Interference studies of FeLV-A, -B, -C and -T have suggested that each subgroup uses a specific receptor in order to infect a host cell. As yet, a receptor for FeLV-A has not been found. FeLV-B and -T use feline Pit 1 as a receptor, FeLV-T requiring a cofactor called FeLIX, an endogenous protein highly related to the N-terminal portion of FeLV envelope protein [Takeuchi *et al.* 1992; Anderson *et al.* 2000]. Pit 1, also the receptor for gibbon ape leukaemia virus, is a multiple membrane spanning protein with a sodium-dependent phosphate transporter function [Kavanaugh *et al.* 1994]. In addition, some FeLV-B isolates have been shown to use feline Pit 2 as a receptor as well as Pit 1. Feline Pit 2 is a phosphate transporter protein related to Pit 1, with approximately 53% homology at the amino acid level. This finding has led to the suggestion that subsequent evolution of FeLV-B by recombination may lead to this dual receptor use, facilitating viral entry and replication [Anderson *et al.* 2001]. Recently, the receptor for FeLV-C has been cloned and found to be a member of the major-facilitator superfamily of transporters, possibly transporting an organic anion [Quigley *et al.* 2000; Tailor *et al.* 1999]. Studies have shown that cells otherwise resistant to FeLV-C may become infected with the virus if the receptor to FeLV-C is over expressed [Tailor *et al.* 2000].

1.2.3.2 Penetration and uncoating

The way in which the retroviral particle enters the cell is not well understood. It is generally thought that after binding, the viral envelope and the cell membrane fuse to release the virion core into the cytoplasm. Two distinct processes have been put forward for the way in which this occurs. Evidence suggests that in the case of HIV, fusion probably occurs immediately after binding as cells expressing HIV Env protein fuse directly to uninfected cells expressing its receptor [Lifson *et al.* 1986]. Other retroviruses, however, are possibly internalised due to receptor-mediated endocytosis with subsequent fusion of the viral envelope and endosomal membrane.

1.2.3.3 Reverse transcription of genome RNA into DNA

Once the retroviral core has gained access to the cell, the virion reverse transcriptase makes use of a proline tRNA primer to initiate DNA synthesis [Baltimore, 1970]. Firstly, a negative-strand DNA molecule is transcribed which is then used to derive the positive-strand DNA. The reverse transcriptase also carries a ribonuclease H function which produces degradation of the virion RNA and removal of the tRNA primer. Generation of the LTRs occurs by duplication of sequences at the 3' (U3) and 5' (U5) poles of the RNA genome [Fields *et al.* 1990].

It has been suggested that DNA synthesis takes place in a structure derived from the viral capsid [Bowerman *et al.* 1989], and that a p27 and viral DNA complex is important in DNA integration [Brown *et al.* 1987]. Whilst the cellular location of DNA synthesis has yet to be fully clarified, cell fractionation studies suggest that it takes place within the soluble cytoplasmic fraction [Varmus *et al.* 1982].

Once cDNA of the viral genome has been synthesised, it becomes closely bound to or contained within the nucleus. As well as the linear form, there is also circular viral DNA produced from either ligation of the linear form or defective DNA generated in the replication process.

1.2.3.4 Integration

Integration involves the insertion of newly synthesised linear viral cDNA into host DNA. During the integration process, both the viral DNA and the host DNA undergo characteristic modifications, consisting of various base deletions and duplications, which have been found to be virtually universal in this family of viruses.

The mechanism of this process has been studied using avian leukosis virus (ALV) and MuLV. Data from these studies suggest that integration is mediated by a ribonucleoprotein complex derived from the viral core [Bowerman *et al.* 1989]. These complexes have been found to contain linear and circular forms of DNA. In the case of MuLV, low levels of circular DNA produced from provirus integrating to itself or LTR circles have been detected *in vitro* and have also been detected to a lesser extent *in vivo* [Shoemaker *et al.* 1980].

Experiments *in vitro* show that the 3' viral DNA adjoins the 5' cellular DNA while the 5' viral DNA remains unattached. This is strong evidence that a linear integration intermediate is formed as opposed to circular DNA containing two LTR sequences [Fujiwara *et al.* 1988]. Ligation of the 5' viral DNA is completed by cell repair mechanisms.

Experiments on MuLV show that integration does not require the use of ATP [Brown *et al.* 1987]. This suggests the possibility of topoisomerase catalyst type reaction rather than cleavage and ligation. The catalyst of the integration reaction is integrase protein encoded by the *pol* gene. In the case of FeLV, this enzyme produces a staggered cut in the host DNA and a four base pair duplication at the insertion site.

Upon integration the 5' and 3' ends of retroviral DNA are always 5'-TG.....CA-3' which implies that these dinucleotides are somehow crucial to the integration process [Colicelli *et al.* 1988]. The location at which the viral DNA inserts into the cellular DNA appears to be a more random process. Certain studies show no strong sequence similarity around areas of integration [Robinson *et al.* 1986], whereas others suggest more specificity. For example a study of integration sites of ALV into avian cell DNA demonstrated that 20% of integrations inserted into one of around a thousand sites

[Shih *et al.* 1988]. Other work has suggested that integration frequency is higher in transcriptionally active areas that have an "open" chromatin structure, thereby being more accessible [Rohdewohld *et al.* 1987].

Once the provirus has been integrated it is then stable from excision or translocation. Any loss or destruction of provirus is due to random cellular processes or recombination, often between the LTRs of the provirus.

1.2.3.5 Proviral expression

The subsequent transcription and translation of the provirus is carried out entirely by cellular mechanisms. The LTR sequences provide signals to the cellular machinery to encourage efficient expression. As with cellular mRNA, retroviral genomes are synthesised by RNA polymerase II. In FeLV, full-length virion RNA produced functions as mRNA for the production of *gag* and *pol* gene products, whereas Env proteins are translated from a 3kb spliced sub-genomic RNA.

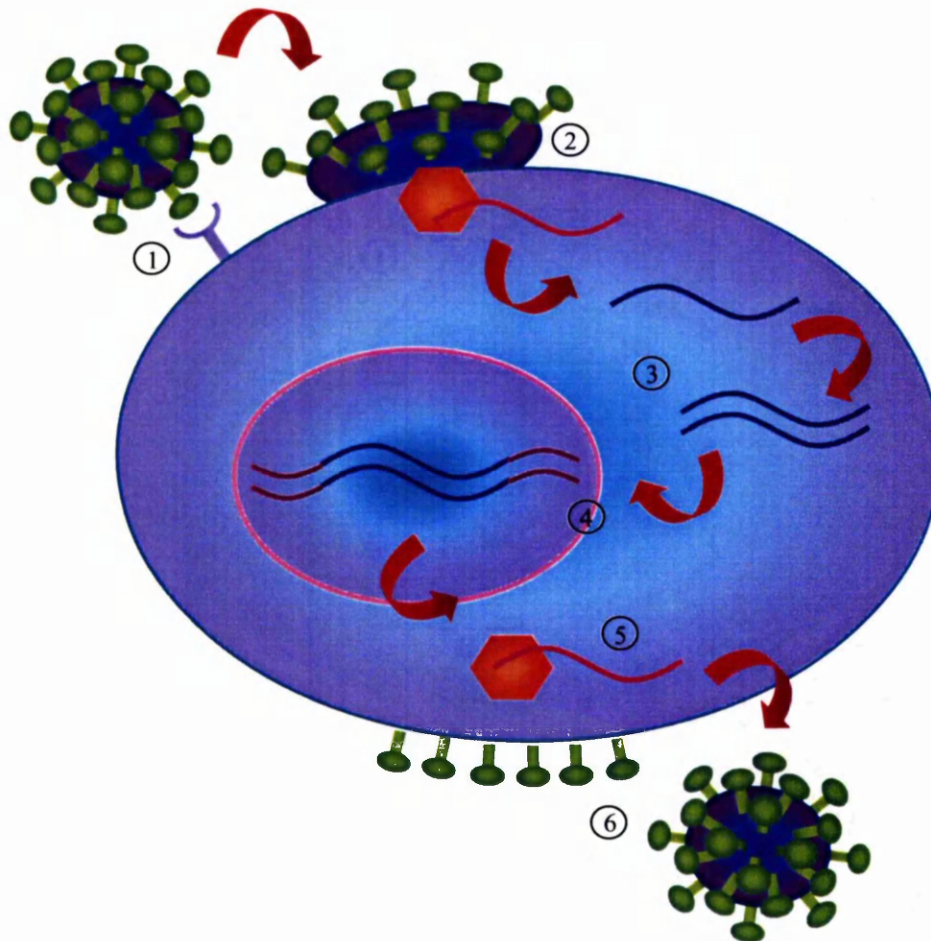
During translation, the full length mRNA is responsible for the production of two main products, a Gag precursor protein and a Gag/Pol precursor. Similarly, an Env precursor protein is synthesised from the spliced fragment of genomic RNA. These protein products make up the entire structure of the virion. In addition, the *gag* gene is also expressed in a different glycosylated form and is both released from the cell and found on the cell surface. This protein is not required for *in vitro* viral expression and its function is as yet unknown. However, some have suggested that it plays a role in immunoevasion by inducing immunotolerance of the host to Gag proteins [Jarrett, 1999].

1.2.3.6 Virion assembly and budding

Once the mature components of the virion have been produced, they are assembled at the host cell membrane where budding of the virus particles takes place. The envelope of the virion consists of host-cell membrane studded with virus-coded surface spikes composed of gp70 and p15E proteins. Just beneath the cell surface the capsid is assembled and rearranged to a condensed form just before budding takes place.

During the budding process, virion aspartyl protease cleaves Gag and Gag/Pol precursors into the mature Gag and Pol proteins. Similarly, the Env precursor protein is cleaved at budding to yield the mature proteins gp70 and p15E. Finally the virus particle is released from the cell by a process which is usually non-cytopathic.

Figure 1-6 Replication of feline leukaemia virus



This diagram shows the replication of FeLV. The virus first binds to a specific cell-surface receptor on an FeLV susceptible cell (1). The virus is transported across the cell membrane where uncoating takes place (2). The single stranded RNA genome is reverse transcribed into double stranded DNA (3), and integrated into the cellular genome to become a provirus (4). The provirus is expressed using cellular mechanisms (5) and the virus is assembled close to the cell membrane. Budding then takes place where the envelope of the virus is derived from the cell membrane (6).

1.2.4 Classification of FeLV

FeLV is a member of the Retroviridae family of viruses. This family consists of Alpha-, Beta-, Gamma-, Delta- and Epsilonretroviruses as well as Lentiviruses and Spumaviruses. FeLV is classified as a Gammaretrovirus. An older classification system is based on viral morphology where retroviruses may be distinguished by differences in core structure and surrounding membrane [Bernhard *et al.* 1958]. In this case, FeLV is classified as a C-type retrovirus.

FeLV consists of three major subgroups, FeLV-A, FeLV-B and FeLV-C. Classification of these groups is reviewed by Sparkes [1997]. The subgroups were distinguished originally by studies of viral interference patterns and neutralisation tests [Sarma and Log, 1973]. In feline fibroblast cells *in vitro*, different virus subgroups prevent superinfection with other viruses of the same subgroup. Subsequent studies have shown that subgroups may be distinguished by *env* sequences, which encode the N-terminal region of gp70 surface glycoprotein [Riedel *et al.* 1988; Mullins *et al.* 1990]. More recently, another subgroup, FeLV-T, has been isolated which also has unique interference properties and is T-cell tropic, infection being limited to feline T-cells and feline fibroblasts [Moser *et al.* 1998; Rohn *et al.* 1998].

All cats naturally infected with FeLV harbour FeLV-A, the dominant subgroup [Jarrett *et al.* 1978a]. It has been reported that 50% of naturally infected cats are infected with FeLV-A alone, 49% with FeLV-A and B and 1% with any combination containing FeLV-C [Jarrett *et al.* 1978a; Rojko and Hardy, 1994].

FeLV-A has been shown to be readily transmissible, although the proportion of cats becoming viraemic is age-dependent. Of the three subgroups FeLV-A is the least pathogenic, being slow to cause disease and frequently resulting in latent infection [Hoover *et al.* 1991; Rojko and Kociba, 1991]. The sequence of the FeLV-A genome is highly conserved [Donahue *et al.* 1988], and the protein structure of FeLV-A Env protein is similar for all FeLV-A isolates. Virus neutralising antibodies to FeLV-A gp70 (SU) are therefore cross-reactive across the subgroup [Russell and Jarrett, 1978a; Donahue *et al.* 1988]. The presence of such antibodies in an infected cat

correlates highly with an absence of active viraemia with FeLV [Russell and Jarrett, 1978b].

FeLV-B arises by recombination between FeLV-A sequences and endogenous FeLV-related sequences (enFeLV). enFeLV are incomplete proviral retrovirus sequences which occur in all domestic cats [Soe *et al.* 1985; Rojko and Hardy, 1994]. Studies suggest that FeLV-C is derived from FeLV-A by mutation, although recombination remains a possibility [Rigby *et al.* 1992]. This recombination hypothesis came from studies using specific probes from the *env* genes of the FeLV subgroups as most sequence differences were found here. Probes of FeLV-A *env* detected FeLV-A and C isolates, and probes of FeLV-B detected independent FeLV-B isolates and endogenous FeLV-related sequences [Stewart *et al.* 1986].

FeLV-B and -C subgroups possess variants, which may be replication-competent or replication-defective. Replication-defective viruses require the additional presence of FeLV-A in order to provide functions that have been lost through mutation or recombination [Stewart *et al.* 1986; Overbaugh *et al.* 1988]. It is thought that these subgroups rely on phenotypic mixing with FeLV-A where FeLV-B or -C are encapsulated in FeLV-A envelope, facilitating spread of the less transmissible subgroups [Jarrett *et al.* 1973; Rigby *et al.* 1992; Rojko and Hardy, 1994]. The fact that FeLV-B and -C arise from mutation or recombination events of FeLV-A and may rely on FeLV-A for replication, is consistent with the fact that FeLV-A can always be isolated from individuals infected with subgroups B and C [Rojko and Hardy, 1994]. Subgroups B and C have not been shown to transmit horizontally on their own. It is likely that co inoculation with FeLV-A would be necessary for successful infection [Jarrett *et al.* 1978; Jarrett *et al.* 1984; Hoover *et al.* 1991].

A recent study has suggested that FeLV-B may inhibit FeLV-A infection possibly due to an immune mediated mechanism. The proportion of cats developing chronic viraemia from inoculation with FeLV-A alone was decreased with co inoculation with FeLV-B virus [Phipps *et al.* 2000].

1.2.5 Transmission of FeLV

Cats infected with FeLV excrete the virus mainly via the saliva and nasal secretions. FeLV has also been found to replicate in the mucosal epithelium of the intestine and urinary bladder, but this is not a major source of infection, as the virus does not live long in the urine or faeces [Hoover *et al.* 1977]. FeLV is therefore transmitted through socialising of infected cats with susceptible individuals [Hardy *et al.* 1973a; Jarrett *et al.* 1973]. Persistently infected cats secrete high titres of infective virus in the saliva generally five times that found in the plasma, although survival time outwith the cat is less than two hours [Francis *et al.* 1977]. Therefore FeLV is transmitted most effectively by direct contact or communal feeding or watering. Indirect contact such as aerosol or infection from the surrounding environment has not been found to be important [Hoover *et al.* 1977; Francis and Essex, 1980].

Venereal transmission of FeLV may occur as the virus has been detected both in the epithelium of the urogenital tract, semen, vaginal fluids and the virus has also been shown to be transmitted to the foetus across the placenta [Hardy *et al.* 1973a; Rojko *et al.* 1979a]. One study indicated that exposure through the milk is a higher risk. Progeny of an infected cat were shown to contract the virus from 45 days after birth when FeLV neutralising antibodies could no longer be isolated from the milk. FeLV antigen and virus were detected in the milk, making it likely that transmission was via this route [Pacitti *et al.* 1986].

1.2.6 Pathogenesis of FeLV

The outcome of infection with FeLV depends on the ability of the host to mount an effective immune response. There are various possible disease states that may occur from infection with this virus: acute infection, transient viraemia without latent infection, transient viraemia with latent infection, atypical infection and persistent viraemia. These outcomes are summarised by Sparkes [1997], and Hoover and Mullins [1991] (figure 1.7).

1.2.6.1 Acute infection

Oronasal exposure to FeLV allows infection of B and T lymphocytes and macrophages present in lymphoid tissue, primarily the tonsils. The virus may subsequently infect myeloid and erythroid haematopoietic progenitor cells in the bone marrow bringing about a cell associated viraemia [Rojko *et al.* 1979a; Hoover *et al.* 1980; Rojko and Kociba, 1991].

1.2.6.2 Transient viraemia without latency

Most adult cats exposed to FeLV undergo transient viraemia that lasts up to 12 weeks whereby virus replicates and is shed. If these cats develop an immune response before infection becomes established in the bone marrow, then the virus is eliminated and no latent infection occurs. Virus neutralising antibodies can usually be detected in the blood in these individuals [Madewell *et al.* 1983]. Susceptibility of adult cats to FeLV may be enhanced if the immune system is compromised, for example in treatment with adrenal corticosteroids [Rojko *et al.* 1979b].

1.2.6.3 Transient viraemia with latent infection

In some cats, protective immunity occurs after the virus has reached the bone marrow. This results in a transient infection lasting several weeks with the subsequent development of immunity. However in 30 - 70% of cats, latent provirus persists in the bone marrow, being held in check by the immune response [Post and Warren 1980]. It is thought that animals at this point do not shed virus, as cats exposed to latently infected individuals fail to show signs of exposure [Madewell *et al.* 1983]. Most latently infected cats have been found to completely eliminate provirus from the bone marrow within 30 months of exposure to FeLV [Pedersen *et al.* 1984]. However studies have shown that a small proportion, around 10%, maintains latency for a prolonged period of time. Loss of latent virus is thought to occur because the infected immature marrow cells eventually differentiate to extinction, or are removed by the immune response [Pacitti *et al.* 1985]. Those cats that do remain latently infected are at risk of reactivation of infection if their immune system is sufficiently compromised, such as administration of adrenal corticosteroid hormones [Rojko *et al.* 1979b].

However it is unlikely that latent infection plays a significant role in pathogenesis since it has been shown that cats who recover from natural FeLV exposure have a similar fatality rate to those who have never been exposed to the virus [McClelland *et al.* 1980].

1.2.6.4 Atypical infection

Atypical infection occurs in less than 5-10 % of cats exposed to virus. In these cases, neither virus nor antigen can be detected in the blood, but FeLV can be found sequestered at various sites. These individuals can exhibit intermittent clinical signs of infection and subsequently either eliminate the virus or become persistently infected in the future [Hayes *et al.* 1989; Rojko and Hardy, 1994].

1.2.6.5 Persistent viraemia

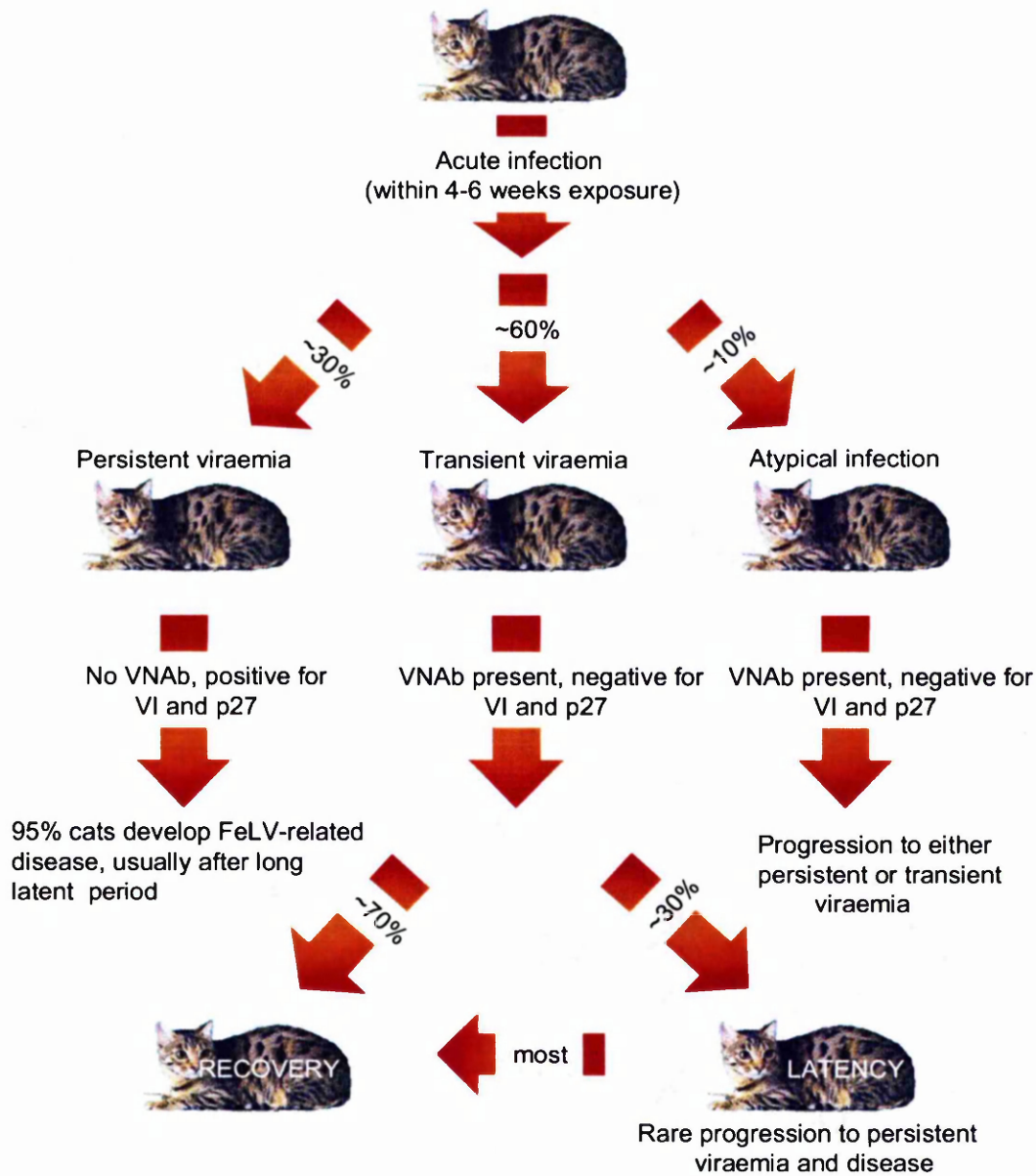
After the initial acute infection, the infected cat may become viraemic depending on whether an immune response can control and eliminate the virus. If the response is insufficient to prevent the virus becoming established in the bone marrow, then persistent viraemia will occur. Bone marrow infection generally occurs two to six weeks after viral exposure. At this point provirus becomes integrated into cells of the bone marrow and viral replication takes place. On dissemination by the blood, the virus replicates in cells undergoing cell division producing localisation in mucosal and glandular epithelial cells of the pharynx, nasal passages, trachea, oesophagus, urinary bladder, intestines, salivary glands and pancreas [Rojko *et al.* 1979a]. Persistently infected cats are highly contagious and virus is released from the epithelium in large quantities into the local environment [Hardy *et al.* 1973a; Jarrett *et al.* 1973].

Viral replication in cells of the bone marrow over long periods, will give rise to multiple integration of provirus into host cells [Mullins *et al.* 1980]. Continual replication in haemolymphatic tissues brings about depletion in lymphoid and myeloid cells and gradual immunosuppression. In time, malignant cell transformation may eventually occur [Hoover *et al.* 1980; Neil *et al.* 1987; Mullins *et al.* 1990]. The

generation of more pathogenic FeLV variants FeLV-B and FeLV-C may also occur at this point [Neil *et al.* 1987; Mullins *et al.* 1990].

The clinical signs of disease are often not displayed in persistently infected individuals for periods of months to years. During this time they remain viraemic and infectious to other cats. Persistent infection is rarely reversed and almost all cats will eventually succumb to FeLV-associated diseases later in life, often around 2-4 years of age [Hoover *et al.* 1991]. It is presumed that most cats succumb to disease at this age, because animals are most susceptible to infection in the early months of life and the sequence of events from infection to clinical disease requires an approximate 2-4 year incubation period.

Figure 1-7 Outcome of FeLV infection [Hoover and Mullins, 1991].



1.2.6.6 Susceptibility of the host to FeLV infection

The most crucial factor affecting susceptibility to FeLV infection is the potency of the immune system. Also important is the viral dose, the duration of exposure, the strain of virus and the age of the cat. Increasing maturity has shown to correlate with increased resistance to FeLV, cats up to 12 weeks of age being highly susceptible to infection [Hoover *et al.* 1976]. Once the mature immune system has developed, cats

tend to recover from transient viraemia and by the age of 16 weeks they are difficult to experimentally infect. As already explained, compromising the immune system with steroid treatment can break down this resistance and leave individuals susceptible to infection [Rojko *et al.* 1979b]. It is a strong possibility that genetics affect susceptibility to FeLV, particularly the type of histocompatibility complex inherited, but this has yet to be proven [Winkler *et al.* 1989].

1.2.7 Diseases associated with FeLV infection

The clinical consequences of FeLV infection can be broadly split into two categories: acute and chronic. Cats that contract the virus early in life may develop viraemia followed by an acute immunodeficiency syndrome with possible mortality within a year. Those cats that are asymptomatic during a prolonged incubation period of months to years may develop a chronic immunodeficiency syndrome. These cats will have an increased risk of tumour development.

1.2.7.1 Acute FeLV infection in the young cat

Studies of cats neonatally infected with FeLV have shown an impeded growth rate and higher death rate than control kittens, many dying between 8-12 weeks of age. On necropsy, thymic atrophy and lymphoid depletion are usually present. Other infections may be evident which may have been a consequence of FeLV infection. Some may also develop alimentary lymphosarcoma or lymphoblastic leukaemia [Anderson *et al.* 1971]. One group has shown what they describe as an acute feline immunodeficiency syndrome, characterised by a survival period of less than 180 days in cats inoculated at less than 8 weeks of age [Hoover *et al.* 1987]. In this syndrome a short asymptomatic period of 2-10 weeks was followed by the onset of disease: progressive lymphocytopenia, decreased lymphocyte blastogenesis, loss of weight resulting in emaciation, persistent diarrhoea and opportunistic infections. Infections included bacterial rhinitis, pneumonia and necrotising stomatitis.

1.2.7.2 Diseases associated with chronic FeLV infection

These diseases are summarised by Hoover and Mullins [1991]. They consist of cytoproliferative diseases including lymphosarcoma and FeLV-negative lymphosarcoma, leukaemia, myeloproliferative disorders, fibrosarcomas, immunosuppressive disease, myelosuppression, anaemia, and leucopenia. Less prevalent are enteropathy, infertility syndrome and neurologic disorders.

Lymphosarcoma

Lymphosarcoma is the most common form of neoplasia associated with FeLV [Hardy *et al.* 1976] and it is likely that in time, all FeLV isolates would induce this disease provided another fatal FeLV-associated disease did not occur first. There are a significant proportion of cats with lymphosarcoma where FeLV cannot be isolated, although in these cases there is a correlation of FeLV exposure, suggesting it could be the aetiological agent [Hardy *et al.* 1980].

Although in many cases the molecular basis for the induction of lymphosarcoma is unknown, two modes of genetic mutation have been identified: transduction and insertional mutagenesis. Transduction is a process of recombination between retroviral DNA and cellular oncogenes to produce an oncogenic variant. Diseases caused by recombinant viruses tend to be restricted to a single host probably due to a "swamping" effect by high titres of FeLV virus in the host. Many cellular genes have been shown to be transduced by FeLV including *fes*, *fms*, *abl*, *sis*, *kit* and *myc* genes. Neil *et al.* [1987], has reviewed the role of FeLV in naturally occurring leukaemias. Several isolates of FeLV/*v-myc* provirus were found originating from a recombination event between viral and cellular genes [Neil *et al.* 1987]. In one study, transduction with *c-myc* gene was responsible for 21% of 63 lymphoid tumours and was the most frequently mutated gene (32%) [Tsatsanis *et al.* 1994].

Secondly, proviral insertional mutagenesis can bring about the onset of neoplasia in FeLV. In the case of lymphoma, several oncogenes and integration loci have been described including *fit-1*, *flvi-1*, *flvi-2*, *c-myc* and *pim-1*. Tsatsanis *et al.* described the frequency of proviral insertion as *flvi-2* (24%), *c-myc* (11%), *fit-1* (8%), and *pim-1*

(5%). Of the tumours examined, 19% were mutated at two sites, and 5% at three of the sites mentioned [Tsatsanis *et al.* 1994].

In addition, mutation within the LTR region has been detected in thymic lymphosarcomas where duplications of sequence in the core enhancer domain often occur [Granoff and Webster 1999].

Three anatomic groups of feline lymphosarcoma have been found [Mackey *et al.* 1972a; Mackey *et al.* 1972b]. In thymic lymphosarcoma the mass is found in the cranial mediastinum and is often associated with an effusion of neoplastic cells in the thorax, which may cause dyspnoea. The neoplasia is usually solitary and tends to occur in cats under 3 years of age. Multicentric lymphosarcoma lesions occur in the lymph nodes and other organs. The clinical signs associated with this are morbidity, anaemia, anorexia and debilitation as well as signs relating to space occupying lesions. Lastly, alimentary lymphosarcoma exhibits lesions in the intestines, mesenteric lymph nodes, kidneys and liver. Clinical signs demonstrated here are usually connected with the intestines and renal function. In addition, lymphosarcoma cases often show immune dysfunction prior to neoplasia [Perryman *et al.* 1972].

Leukaemia and myeloproliferative disorders

Leukaemia occurs through the transformation of haemopoietic cells in the bone marrow, which are then released into the circulation. This may produce an increase in total leukocyte count. The clinical signs associated with leukaemia include weight loss, lethargy, pallor, listlessness, anaemia, intestinal disruption, hepatosplenomegaly and opportunistic infections. The most common leukaemias of cats are erythroid and myelomonocytic leukaemia [Fraser *et al.* 1974; Facklam and Kociba, 1986].

Myeloproliferative disorders display an abnormal growth and differentiation of haemopoietic cells within the bone marrow. It is similar to a subleukaemic stage of leukaemia. The clinical signs are similar to leukaemia, but animals also have a nonregenerative, macrocytic anaemia [Ward *et al.* 1969; Blue *et al.* 1988].

Fibrosarcoma

Multicentric fibrosarcomas in young cats may be caused by feline sarcoma viruses (FeSV) which are recombinants between FeLV and cellular proto-oncogenes. During this process some of the viral genome is lost, so FeSV must be complemented by a concomitant FeLV-A infection in order to replicate [Besmer *et al.* 1983]. The neoplasia may be lethal or may regress completely over time.

Immunosuppressive disease

It has been shown that all FeLV subtypes cause dysfunction of the immune system due to the development of T- and B-cell deficits [Cockerell *et al.* 1976; Trainin *et al.* 1983], which usually begin within the first two months after infection. Specific FeLV subtypes tend to display similar patterns of progressive immunosuppression. FeLV-A causes a slow gradual deficiency, which may suddenly worsen with the development of variants such as FeLV-C [Jarrett *et al.* 1984; Mullins *et al.* 1991].

Studies have shown that some variant viruses are more acutely pathogenic than others. It has been demonstrated that small genetic substitutions in *env* gene of one isolate in a family of variants caused changes in function, structure and antigenicity of SU and TM protein which is responsible for its increased T-cell cytopathogenicity [Overbaugh *et al.* 1988; Poss *et al.* 1989]. In addition, some groups have shown that the TM protein itself may have an immunosuppressive effect [Mathes *et al.* 1979].

Suppression of the haemopoietic system

Suppression of the haemopoietic system brings about a group of diseases depending on the cell type affected, including myelosuppression, anaemia and leucopenia. Myelosuppression, or suppression of cells of the bone marrow, may produce clinical signs such as weight loss, anorexia, anaemia and secondary infection. If the erythroid cells are predominantly affected, then anaemia and its associated clinical signs will prevail, as will leucopenia if leucocyte suppression is the major effect.

It is thought that the virus genotype or mix of genotypes will dictate the degree of myeloid versus erythroid suppression involved. The most specific link between FeLV

subtype and disease is FeLV-C and pure red cell aplasia [Jarrett *et al.* 1984; Dornsife *et al.* 1989]. The anaemia associated with FeLV-C is usually nonregenerative, normocytic and normochromic with no significant reticulocytosis, whereas the more general anaemia produced by other subtypes is often more varied [Cotter *et al.* 1979; Weiser and Kociba, 1983].

Studies have shown that the anaemogenicity of FeLV-C Sarma is localised to the N-terminus of SU protein [Riedel *et al.* 1986; Riedel *et al.* 1988;]. Other work has demonstrated that FeLV-C infects marrow erythroid progenitor cells preventing proliferation and differentiation, leading to impaired red blood cell production [Boyce *et al.* 1981; Abkowitz *et al.* 1987].

Enteropathy

FeLV can infect the crypt cells of the intestinal mucosa causing chronic diarrhoea and weight loss. This is characterised histologically by crypt cell damage and villus atrophy and fusion [Reinacher *et al.* 1987].

Foetal resorption

Infertility relating to foetal resorption has been shown to occur where conception and implantation occurs in viraemic animals, but within the first month of gestation the foetuses die and become autolysed. Sometimes, the foetus may be carried to term and persistently infected kittens are born [Cotter *et al.* 1975; Hoover *et al.* 1983].

Neurologic disease

A small number of FeLV infected cats develop neurological dysfunction such as polyneuropathy, locomotory disorders and lower motor neurone paralysis [Haffer *et al.* 1987; Mitchell *et al.* 1997].

1.2.8 Immune response to FeLV

The mechanism by which immunity to FeLV is achieved has yet to be fully elucidated, but work has concentrated on virus neutralising antibodies (VNAb), antibody to other viral antigens, anti-feline oncornavirus associated cell membrane antigen (FOCMA) antibodies and cell mediated immunity.

1.2.8.1 Virus neutralising antibodies and antibodies to other antigens

Virus neutralising antibodies to FeLV are directed at the envelope proteins of the virus. Immune cats produce a strong antibody response to SU and TM envelope protein preventing attachment and binding of the virus to the cell specific receptor of the host [Lutz *et al.* 1979; Rojko and Olsen, 1984; Rojko and Kociba, 1991]. There is a strong correlation between these high VNAb titres and resistance to infection. Immune cats do not normally produce antibodies to Gag proteins. It has been suggested that naturally infected cats are transiently anergic to these proteins. Since cats that are inoculated with recombinant protein produce anti-Gag antibodies [Charreyre and Pedersen, 1991], a theory has been proposed that FeLV has evolved a way of inducing anergy to prevent a CTL response to Gag-expressing cells [Jarrett, 1999]. This may be due to the way in which Gag proteins are glycosylated.

The active development of VNAb has been shown to be associated with resistance to FeLV infection [Hardy *et al.* 1976]. It has also been shown that passive transfer of VNAb from the milk of the dam to her offspring will protect the kittens from infection [Jarrett *et al.* 1977]. However, VNAb are not essential for protection against FeLV and VNAb may be found in persistently viraemic cats [Charreyre and Pedersen, 1991; Rojko and Hardy, 1994]. This has led to investigation into antibodies that are produced to other proteins such as NC, MA, TM, CA and other SU antibodies [Lutz *et al.* 1980; Snyder *et al.* 1985]. These studies suggest that antibodies other than VNAb are found at a higher level in resistant individuals than those persistently viraemic, suggesting a possible role in immunity against FeLV.

1.2.8.2 Anti-FOCMA antibodies

Feline oncornavirus-associated cell membrane antigen (FOCMA) is a protein which is expressed on all cells that have been infected with FeLV. Antibodies to FOCMA have been shown to be cross-reactive with antibodies to the SU protein of FeLV-C/Sarma [Vebrat *et al.* 1983]. This has led to the hypothesis that FOCMA may be endogenous FeLV-related sequences expressed as an antigen on the cell surface. Some studies have shown that the occurrence of anti-FOCMA antibodies is associated with protection against some FeLV-induced neoplasia [Rojko and Kociba, 1991; Rojko and Hardy, 1994]. Cats with leukaemia or lymphosarcoma have been shown to have negligible or low anti-FOCMA antibody titres, whereas cats with high titres appear to be protected even though they are viraemic [Cotter *et al.* 1974; Essex *et al.* 1976]. The mechanism by which this occurs may be associated with a complement-dependent reaction to transformed cells bringing about lysis [Grant *et al.* 1979].

1.2.8.3 Cell-mediated immunity

As VNAb have been shown not to be essential for protection against FeLV, this has led to the idea that cell mediated immunity may play an important role in resistance [Jarrett, 2001]. This hypothesis is supported by work showing that a DNA vaccine to FeLV demonstrated a high degree of protection without the detection of anti-FeLV antibodies following immunisation [Hanlon *et al.* 2001]. Those cats protected in this study were shown to have higher FeLV-specific CTL responses in blood and lymphoid organs than those who were not vaccine immune [Flynn *et al.* 2000a]. Further to this, CTLs were shown to appear before VNAb in recovering cats whereas those with persistent viraemia displayed a silencing of FeLV-specific humoral and CTL responses [Flynn *et al.* 2002].

1.2.9 Diagnosis of FeLV infection

In persistent viraemia, virus may be detected in sites of replication such as the bone marrow and saliva, as well as in plasma and neutrophils of the blood [Hoover *et al.* 1977]. The blood may be used as an indicator of infection in several ways. Plasma can

be used for virus isolation (VI), or a blood smear can be used to detect p27 antigen in neutrophils by immunofluorescence [Hardy *et al.* 1973b]. Studies have shown that a persistently infected cat will give a positive result for both of these techniques [Jarrett *et al.* 1982].

In addition, during viraemia infected cells produce large amounts of p27 antigen which is released into the plasma. This has allowed the use of a capture enzyme-linked immunosorbent assay (ELISA) for p27 detection in plasma. The first commercial ELISA came on the market in 1979 (Leukassay F; Pitman-Moore) [Hartmann *et al.* 1973]. This was a sensitive detection system, but not very specific and has since been improved. There are many "in practice" detection kits for p27 also available which are based on ELISA or rapid immunomigration (RIM) [Hartmann *et al.* 2001]. Some studies have compared the use of RIM and p27 ELISA and found discrepancies due to differential sensitivity and specificity. Thus, it is usually recommended that these tests are confirmed by VI or immunofluorescence [Robinson *et al.* 1998].

In addition, "discordant" cats are positive for FeLV antigen by ELISA but are negative by VI [Jarrett *et al.* 1991]. Up to 10% of cat plasma samples that are antigen-positive do not contain infectious virus. Virtually all these cats were from households with FeLV infection so it is possible that cats were sampled at the beginning or the end of an infection. In some, however, this discordant state persisted for a long period of time. It has been suggested that these individuals could have had focal or atypical infection, which releases antigen but not virus into the blood. Cats in this state tended to eventually become seronegative to both tests, but some reverted to viraemia even after long periods, so these individuals should probably be considered a risk of infection to other cats.

On rare occasions cats have been found to be negative for p27 antigen but positive for virus isolation [Jarrett, 1991]. This situation could be due to a mutant FeLV that either fails to release high levels of p27 into the plasma or produces a mutant p27 molecule that cannot be detected by the monoclonal antibody used to capture p27 in the ELISA.

1.2.10 Control of FeLV infection

In order to control the spread of FeLV in a household, persistently infected cats must be identified and separated from FeLV negative cats. The household must then be quarantined and no cats may be taken into or out of the area. All equipment associated with the cats should be disinfected with diluted household bleach to kill the virus. It is recommended that all cats be re-tested after twelve weeks. The reason for this is to ensure that the positive cats are persistently viraemic rather than undergoing transient infection and also to ensure that virus negative cats were not incubating infection at the time of testing. The negative cats at this point are considered FeLV free and should be re-tested every six to twelve months. Quarantine is then terminated, but any cats brought into this environment should be tested beforehand and kept in isolation until the test twelve weeks later has proven to be negative. In the case of positive cats, these should either be euthanased or kept in isolation. These animals should not be used for breeding, as it is likely that all progeny will be FeLV positive [Hardy *et al.* 1976].

CHAPTER 2: MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Materials

Any materials specific to given chapters are described in the relevant sections.

2.1.1 General

2.1.1.1 Major equipment

- Sequencing apparatus and appliances: Licor 4000 (Licor Incorporated, USA), ABI Prism 3100 genetic analyzer (Applied Biosystems, Warrington, UK).
- Ultraviolet camera system: Photometrics GmbH Sensys, A2S / Leica system (Munich, Germany).
- Benchtop orbital shaker SO3 (Stuart Scientific, UK).
- C25 incubator shaker (New Brunswick Scientific, USA).
- Cell freezer: Model Cryo 10 Series II (Planer products Ltd., UK).
- Gel documentation system: (Ultraviolet products Inc., USA).

Centrifuges

- Microfuge 5415C (Eppendorf GMBH, Germany).
- Benchtop centrifuge GS-6R (Beckman Instruments Inc., USA).
- Ultracentrifuge J2-21 (Beckman Instruments Inc., USA).
- CO₂ incubators for tissue culture (Leec Ltd., UK and Sanyo, UK).
- Pipetteman (P10, P20, P100, P200, P1000): (Gilson Medical Electronics, France).
- Spectrophotometer model DU640 (Beckman Instruments Inc., USA).
- Thermal Cyclers: GeneAmp 9600 (The Perkin Elmer Corporation, USA), PCR Express (Hybaid Limited, Middlesex, UK).
- ABI Prism 7700 sequence detection system (Applied Biosystems, Warrington, UK).
- Vacuum Dessicator (Jencons-PLS, UK).
- Water baths (Grant Instruments Ltd., UK).

2.1.1.2 Consumables

- Falcon tubes: 15 and 50 ml (Corning Incorporated, USA).
- Screw top and flip top 1.5 ml Eppendorf and 0.5 ml tubes (Treff LAB, Switzerland).
- Pipette tips, yellow (200 µl) and blue (1000 µl) (Sarstedts, Germany).
- Endotoxin-free Rainin Greenpak filter tip pipette tips 20, 200 and 1000 µl (Anachem, UK).
- 0.2ml PCR tubes (The Perkin Elmer Corporation, USA).
- Syringes of 1, 2, 5, 10, 20 and 50 ml (Becton Dickinson Labware, UK).
- Needles: 18, 21, 23 and 25 gauge (Becton Dickinson, UK).
- Single use syringe filter, 0.2 and 0.45 µm (Sartorius, Germany).
- Disposable, sterile scalpels (Swann Morton, UK).
- Tissue culture disposables: Costar flasks (TRP, Switzerland), multiwell plates and pipettes (Corning Incorporated, USA).
- Cell scrapers (Greiner Labortechnik Ltd., UK).
- Petri dishes, bijoux and universals (Greiner Labortechnik Ltd., UK).
- Lab Tek II chamber slide system - 8 well (Scientific Laboratory supplies, UK).
- Endotoxin-free Eppendorf tubes (Fisher Scientific, UK).
- 2 ml Cryogenic vials (Corning Incorporated, USA).
- Blood tubes: Heparin 2ml, EDTA 2ml, plain 2ml and sodium citrate 2ml (Sarstedt, Germany).

2.1.1.3 Complete kits and specialist reagents

- PCR–Script Cloning Kit (Stratagene, Netherlands).
- Qiaprep Miniprep Kit (Qiagen, West Sussex, UK) was used to isolate up to 20 µg of plasmid DNA from 3 ml LB bacterial culture.
- Qiagen plasmid Mega Kit (Qiagen, West Sussex, UK) was used to prepare up to 2.5 mg of plasmid DNA from 500 ml LB bacterial culture.
- Qiagen plasmid Endofree Giga Kit (Qiagen, West Sussex, UK) was used to isolate up to 10 mg of high-copy plasmid DNA using 2.5 l LB bacterial culture.
- QIAquick gel extraction kit (Qiagen, West Sussex, UK) for purification of DNA and extraction of DNA from agarose gel.

- QIAamp DNA blood mini kit (Qiagen) for the preparation of up to 120 µg DNA from buffy coat or lymphocyte samples.
- TOPO TA Cloning Kit (Invitrogen Life Technologies, Paisley, UK) for the cloning of PCR products with A overhangs, generated by *Taq* polymerase.
- Gentra Purescript RNA isolation kit (Flowgen, Leicestershire, UK).
- Access RT-PCR System (Promega, UK) for reverse transcription and PCR amplification of specific target RNA from total RNA.
- ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Warrington, UK).
- Performa DTR gel filtration cartridge kit (Edge Biosystems, USA).
- Quantikine human IFN γ kit (R + D Systems, USA).
- ECL+ PLUS Western blotting detection system and ECL Hyperfilm (Amersham Pharmacia, UK).
- LipofectAMINE Reagent (Invitrogen Life Technologies, UK).

2.1.1.4 DNA plasmid vectors

- PCR-Script Amp SK(+) cloning vector (Stratagene, UK) is derived from pBluescript II SK(+) phagemid. This plasmid contains an ampicillin resistance gene, a lac promoter, T3 and T7 RNA polymerase promoters and a multicloning site, which includes *Srf*I restriction site.
- pCI-neo Mammalian Expression Vector (Promega, USA).
- pCR®2.1 TA cloning vector (Invitrogen Life Technologies, Paisley, UK) as part of the TOPO TA cloning kit, supplied as linearised DNA with 3' T overhangs.
- pCR®3.1 Eukaryotic TA Cloning® Kit cloning vector (Invitrogen Life Technologies, Paisley, UK).
- pUSE1⁻ , from a mammalian expression series constructed from pCI-neo (Promega), by Dr Derek Bain.
- pEGFP-C1 vector (Clontech Laboratories Inc., UK) containing GFPmut1 variant used to monitor transfection efficiency.

2.1.1.5 Enzymes

- Restriction enzymes and buffers were supplied by New England Biolabs (Herts, UK), Invitrogen (Paisley, UK), or Stratagene (UK).
- PURESRIPT DNase I (Gentra systems, USA).
- *Pfu* DNA polymerase (Promega, USA) for proof reading polymerase function.
- *Taq* DNA polymerase (Qiagen).
- T4 DNA ligase (New England Biolabs, USA).
- Thermosequenase DNA polymerase supplied as a component of the cycle sequencing kit (Amersham Pharmacia, UK).
- Human recombinant caspase I (Calbiochem, UK).

2.1.1.6 Reagents and solutions

Water

Water for the preparation of media and general solutions was purified using a Millipore RO10 system. Ultrapure water for work using recombinant DNA and protein was purified by a Millipore Q50 water purification system (Millipore (UK) Ltd., Watford, UK). Tissue culture grade distilled water was supplied by Invitrogen Life Technologies, UK. For any work using RNA, DEPC treated water was used. This was prepared by treating dH₂O with 0.05% diethyl pyrocarbonate overnight at room temperature. This was then autoclaved for 30 minutes at 120°C to remove DEPC. Endotoxin free water for DNA vaccine preparation was supplied by Sigma and PCR grade water by Invitrogen Life Technologies, UK.

Chemicals

All general chemicals used were of analytical (Analar) or ultra pure grade, supplied by Sigma Chemical Company, (Dorset, UK) or BDH Ltd., (Poole, UK), unless otherwise specified.

Phosphate buffered saline

- Phosphate buffered saline (1 x PBS): 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.3).
- Endotoxin-free PBS (Biowhittaker UK Ltd.).

2.1.1.7 Oligonucleotides

All oligonucleotides were synthesised and supplied freeze dried by the manufacturer MWG-Biotech and were then resuspended in dH₂O for use. Sequencing primers for Licor model 4000 were modified by IRD 800 labelling. Sequencing primers for ABI Prism 7700 system were unmodified. Specific primer sequences are detailed in the appropriate chapters.

2.1.1.8 ELISA

- Coupling buffer: 10 mM sodium bicarbonate, 1 mM EGTA, pH 9.5, Made at 10 x concentrated and stored at 4°C.
- TBS-T: 20 mM Tris, 140 mM sodium chloride, 0.1% Tween-20, pH 7.6, stored at room temperature.
- TMB liquid substrate system (Sigma, UK).

2.1.1.9 Experimental animals

Specific pathogen free (SPF) cats were purchased from a commercial breeding unit and housed at the isolation unit at The University of Glasgow. They were fed a commercial diet and all procedures were performed according to Home Office regulations.

2.1.2 DNA and protein analysis

2.1.2.1 DNA purification

- STET: 8% sucrose, 50 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 5% triton x 100 in dH₂O, filtered (0.22 µm), stored at room temperature.
- lysozyme (Sigma, UK).

2.1.2.2 Molecular size standards for DNA and protein

- DNA: 1 kb DNA ladder (size range 100 base pairs (bp) to 12 kb) (Invitrogen Life Technologies, Paisley, UK).
- PROTEIN: Kaleidoscope Pre-stained Markers wide range (6.7-205 kDa) (Bio-Rad, Herts, UK).
- Perfect protein HRP conjugate Western blot marker (15kDa - 150kDa) (Novagen, Germany).

2.1.2.3 DNA agarose gel reagents

- DNA loading buffer: 50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol, 100 mM EDTA in dH₂O. Stored at room temperature and used at a dilution of 1:10.
- Ethidium bromide: 10 mg/ml stock in dH₂O, working solution at 3 mg/ml with dH₂O, stored away from light.

2.1.2.4 SDS-PAGE reagents

- SDS-PAGE denaturing loading buffer: 62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, stored at 4°C.
- 10 x SDS-PAGE Running Buffer: Tris base 30 g, glycine 144 g, SDS 10 g in 1 litre dH₂O.
- Polyacrylamide solution: Acrylamide/Bis acrylamide stock solution. 30% (w/v) acrylamide, 1.579% (w/v) bis acrylamide, ratio 19:1 (Severn Biotech Ltd.); stored in the dark at 4°C.

- 1 M Tris HCl: 121 g Tris base in 800 ml dH₂O adjusted to pH 6.8 with concentrated HCl and made up to 1 litre with dH₂O.
- 1.5 M Tris: 181.5 g Tris base in 800 ml dH₂O adjusted to pH 8.8 with sodium hydroxide and made up to 1 litre with dH₂O.
- Towbin transfer buffer: 25 mM Tris, 137 mM glycine, 20% methanol to 500 ml with dH₂O.
- 10 x Tris Buffered Saline (TBS): Tris base 24.2 g, NaCl 80.0 g, HCl 38.0 ml pH 7.6, dH₂O to 1 litre.
- TBS-T: 20 mM Tris, 140 mM sodium chloride, 0.1% Tween-20, pH 7.6, stored at room temperature.
- TBE (10 x): 0.9 M Tris-HCl, 0.9 M Boric acid, 25 mM EDTA pH 8.3 diluted to 1 x with dH₂O.

2.1.3 Bacteria

2.1.3.1 Bacterial strains

- Epicurian Coli XL-10-Gold Kan ultracompetent cells (Stratagene, UK).
- MAX Efficiency STBL2 competent cells (Invitrogen Life Technologies, UK).
- TOP10 cells comprising One Shot chemically competent *E. coli* (Invitrogen Life Technologies, Paisley, UK).

2.1.3.2 Bacterial media and supplements

- Ampicillin: 100 mg/ml in dH₂O. Filtered through a 0.22µm filter, aliquoted and stored at -20°C.
- SOC Medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose.
- Luria-Bertani (LB) medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride in dH₂O, autoclaved and stored at room temperature.
- LB-agar: LB medium containing 1.5% (w/v) agar.
- X-gal solution: prepared as 40 mg/ml stock in dimethylformamide, stored at -20°C.

- Isopropyl β -D-1-thiogalactopyranoside (IPTG): 10 mM in dH₂O, stored at –20°C.

2.1.4 Cell culture

2.1.4.1 Cell lines

- 293T cells were kindly supplied by Celia Cannon (Retrovirus Research Laboratories, University of Glasgow).
- FEA and FeLV-A cells were a kind donation from Mathew Golder (Feline Virus Unit, University of Glasgow).
- Lec8 (CHO) and KG-1 cells were purchased from American Type Culture Collection (ATCC).
- MYA-1 cells were kindly supplied by Hayley Haining (Retrovirus Research Laboratories, University of Glasgow).

2.1.4.2 Media

- All media and supplements were purchased from Invitrogen Life Technologies unless otherwise stated.
- FEA and 293T cells: Dulbecco's Modified Eagle Medium (DMEM) with sodium pyruvate, with 1000 mg/l glucose, with pyridoxine.
- LN156 cells: RPMI 1640 medium with L-glutamine.
- MYA-1 cells: RPMI 1640 medium without L-glutamine.
- KG-1 cells: Iscove's Modified Dulbecco's medium with L-glutamine.
- Lec8 cells: Nutrient mixture F-12 (HAM) with L-glutamine.
- Leibovitz medium

2.1.4.3 Supplements

- Foetal Calf Serum (FCS), (TCS Biologicals LTD., Buckingham, UK): heat inactivated, virus screened, mycoplasma screened.
- Horse Serum heat inactivated at 56°C for 1 hour (Invitrogen, UK).

- Penicillin/streptomycin: supplied as a 100 x stock solution of 10,000 units penicillin and 10,000 μg streptomycin per millilitre, used at a final concentration of 100 IU penicillin and 100 μg streptomycin per ml (1 x Penicillin/Streptomycin). Stored at 4°C.
- L-glutamine: supplied 200 mM (100 x) stock solution stored at 4°C.
- Gentamicin: 10 mg/ml in dH₂O.
- Trypsin-EDTA (1 x) in HBSS W/O CA + MG W/EDTA 4NA. Stored at 4°C.
- β -Mercaptoethanol (2-ME) 14.3 M, DNase, RNase and protease free (Sigma, UK).
- Cell lysis buffer for SDS-PAGE: 100 mM Tris HCl pH 6.8, 2% SDS, 20% glycerol in dH₂O with protease inhibitors.
- Polybrene: registered trademark for hexadimethrine, (Abbott laboratories, USA).
- Hydrocortisone succinate (Sigma, UK).
- 10% dimethyl sulfoxide (DMSO) (Analar, BDH Ltd).
- Caspase buffer: 100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4.

2.2 METHODS

2.2.1 Plasmid DNA production from bacteria

Bacterial cultures for isolation of plasmid DNA were prepared in LB growth medium, while single colonies of bacteria were grown on agar. LB-agar plates were made by filling a 100 mm Petri dish with approximately 15 ml agar. The presence of an ampicillin resistance gene in all plasmids used allowed selective growth of plasmid containing bacteria. Ampicillin was added to both growth media and agar to a final concentration of 100 µg/ml.

For small scale DNA preparation, single colonies of bacteria were selected using a sterile 200 µl pipette tip and transferred to a bijoux containing 3 ml of LB culture medium. These were incubated overnight at 37°C in an orbital incubator.

For large scale DNA preparation, a single colony was transferred into either 3 ml of LB medium (Qiagen Mega kit) or 8 ml LB medium (Qiagen Endofree Giga kit) and incubated for 6-8 hours at 37°C in an orbital incubator. The culture was then added to the appropriate volume of LB medium and incubated in an orbital incubator overnight at 37°C.

Bacterial cultures were preserved for further use by making glycerols from large-scale DNA preparations. A volume of 200 µl of 80% glycerol was added to 800 µl of fresh culture, vortexed briefly and then stored at -70°C. To recover the bacteria from glycerol stocks, the glycerols were thawed, streaked out onto an LB-agar plate using a sterilised platinum wire and incubated overnight at 37°C.

2.2.2 Purification of minipreparations of DNA

The STET method was used to obtain DNA from small-scale preparations. A 3 ml bacterial culture was grown overnight and 1.5 ml was pelleted by centrifugation at 14000 rpm for 2 minutes. The supernatant was removed using a pipette and the

bacterial pellet was resuspended in 85 µl of STET. 35 µl of 10 mg/ml lysozyme in STET was then added and gently mixed. This was boiled at 100°C for 45 seconds and centrifuged for 15 minutes at 14000 rpm. Precipitated DNA was removed using a wooden toothpick, 120 µl of propan-2-ol added and the sample placed at -70°C for 15 minutes. The DNA was harvested by centrifugation at 14000 rpm for 15 minutes, drying of the pellet using a vacuum pump and elution in 20 µl dH₂O. The DNA was stored at -20°C until further use.

High quality small scale DNA preparations required for cloning or sequencing were made using QIAprep Miniprep kit (Qiagen), according to the manufacturer's instructions. This procedure involves alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica-gel membrane. The membrane is washed of endonucleases and salts and then eluted in 50 µl dH₂O.

2.2.3 Large scale DNA preparation

Larger quantities of DNA were prepared using Qiagen plasmid Mega Kit and Qiagen plasmid Endofree Giga Kit (Qiagen, West Sussex, UK) for up to 2.5 mg and 10 mg of plasmid DNA respectively. Both kits consist of a scaled up version of the QIAprep Miniprep kit (Qiagen).

2.2.4 Digestion of DNA with restriction enzymes

Restriction endonucleases were used for analysis and manipulation of DNA. Typically, 1-2 µg of DNA was digested with 5 units/µg of the appropriate restriction enzyme. 10 x enzyme reaction buffer was used at 10% total volume and the reaction was made up to 20 µl in dH₂O. The reactions were incubated at 37°C for at least 1 hour unless otherwise stated.

For preparation of DNA fragments for use in ligation reactions, between 5 and 20 µg of DNA was digested overnight with the appropriate enzyme and reaction buffer to a final volume of 200 µl in dH₂O.

2.2.5 DNA electrophoresis

Typically, 0.8-1.5% agarose gels were prepared as this produced efficient separation of DNA fragments between 800 and 1500 bp. The agarose gel was made using 1 x TBE and ethidium bromide was added at 0.25 µg/ml. The gels were submerged in perspex tanks using 1 x TBE and the comb removed. Samples mixed with DNA loading buffer were loaded onto the gel alongside 1 kb marker. Gels were run between 80-100 volts for 30-90 minutes and bands visualised using a short wave UV transilluminator. Images were then captured using a gel documentation system.

2.2.6 DNA fragment purification

DNA fragments used for ligation reactions were initially visualised on an agarose gel and excised using a sterile scalpel. DNA purification was performed using QIAquick gel extraction kit, (Qiagen, West Sussex, UK) according to the manufacturer's instructions. The DNA was generally eluted in 50 µl of dH₂O.

2.2.7 Ligation of DNA

In general, 100-200 ng of vector DNA was used per reaction and insert DNA calculated to a molar ratio of 1:9, vector:insert. A ligation mix was prepared along with the DNA using 3 units (3 µl) of DNA ligase, 4 µl of 5 x ligation buffer and dH₂O to a final volume of 20 µl. The reaction was incubated overnight at a temperature of 14°C, which maintains a good balance between annealing of ends of DNA and activity of the enzyme. Ligations performed using commercial kits (e.g. pCR-Script (Stratagene)) were carried out according to the manufacturer's instructions.

2.2.8 Quantification of nucleic acid concentration

2.2.8.1 Spectrophotometer

The spectrophotometer was firstly zeroed by measuring the optical density (OD) of DNA diluent at 260 nm. The DNA was diluted in the appropriate diluent at a 1:100 dilution, transferred to a cuvette and an OD reading taken at 260 nm. The concentration of nucleic acid (NA) in the sample was calculated using the following formula:

$$[\text{NA}] \mu\text{g}/\mu\text{l} = \text{OD}_{260} \times \text{NAF} \times \text{DF}/1000,$$

In this formula, NAF (nucleic acid factor) is 50 for DNA, 40 for RNA and 20 for oligonucleotides. The DF (dilution factor) in this case was 100 [Sambrook *et al.* 1989]. For DNA samples prepared as described, the concentration of nucleic acid in $\mu\text{g}/\mu\text{l}$ was $5 \times \text{OD}_{260}$.

2.2.8.2 Agarose gel

Alternatively, DNA amounts could be estimated by running the sample on an agarose gel. The sample band was compared to bands of a known quantity of 1 kb DNA marker following staining with ethidium bromide and UV transillumination.

2.2.9 Bacterial transformation

Transformation of bacteria was performed using commercial kits according to the manufacturer's instructions. Any modifications to the manufacturer's protocol are listed in the appropriate chapters. Volumes of 50 and 100 μl of transformation were plated onto LB-agar plates containing 100 $\mu\text{g}/\text{ml}$ ampicillin. The plates were incubated overnight at 37°C and colonies were selected and cultured as described previously.

Where cloning vectors contained a lac promoter, application of 25 µl of X-gal to agar plates 30 minutes prior to coating with bacterial culture allowed blue/white colony selection. Cloning into the multicloning sites of these vectors causes disruption of lacZ α expression and these constructs remain white, whilst vectors without insert appear blue on the plate.

2.2.10 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels consist of a stacking buffer that initially concentrates the protein into a narrow band and a separating gel, which separates the proteins according to molecular size. The composition of separating gel is determined by the molecular size of the protein to be separated. Higher percentages separate smaller sized proteins. To detect flexi-IL-12 (75kDa), a 12% gel was used whereas a 15% separating gel was used to detect ILRAP-IL-18 (18kDa). Recipes for stacking and separating gels are described by Sambrook *et al.* [1989].

2 glass plates (10 x 7.3 cm and 10 x 8.3 cm) were separated by 0.75 mm spacers and assembled in a casting stand. The separating gel was poured between the plates to a depth of around 5 cm and was overlaid with Tris-saturated butanol to allow the gel to polymerise with a straight edge. Once polymerised, the butanol was removed, the stacking gel poured and a ten well comb was inserted into the top of the gel. Once completely polymerised, the gel was run on the Mini-PROTEAN II electrophoresis system. The gel plates were attached to the gel holder and transferred to the electrophoresis tank. The holder was immersed in 1 x SDS-PAGE running buffer, the comb was withdrawn and the wells were flushed with running buffer to remove any excess gel fragments.

Most protein samples consisted of transfection products and initially 20 µl of either supernatant or cell lysate were used for each well. These samples were diluted appropriately depending on the visibility bands produced upon immunodetection. A suitable volume (5 µl) of 5 x protein sample loading buffer was added to each sample and they were incubated at 100°C for 5 minutes. The loading buffer contains SDS and β -mercaptoethanol, which reduces proteins to their subunits by reducing disulfide

bonds. The samples were then loaded into the wells alongside a molecular size marker. The gel was electrophoresed at 150 V for 70 to 80 minutes until the bromophenol blue had reached the bottom of the glass plates. The gel was removed from the plates and the stacking gel excised with a sterile scalpel and discarded.

2.2.11 Immunodetection of proteins

This technique involves the electrophoretic transfer of protein onto a membrane, in this work either nitrocellulose or PVDF, using a semidry immunoblotter. The protein is then bound to the membrane and can be probed using immunodetection reagents. The membrane is probed firstly with primary antibody and then secondary anti-IgG antibody, which is coupled with horseradish peroxidase. These protein-antibody complexes are then detected using ECL+ PLUS reagents (Amersham Pharmacia, UK). This system relies on the emission of light due to oxidation of luminol by horseradish peroxidase. This light is then detected by a short exposure to blue-light Hyperfilm ECL (Amersham Pharmacia, UK).

Following electrophoresis, the gel was submerged in Towbin transfer buffer along with two similarly sized sheets of extra thick filter paper (Whatman international Ltd., England). A suitably sized Hybond-ECL PVDF or nitrocellulose membrane was pre-wetted in 100% methanol before being submerged in Towbin buffer. A filter paper/membrane/gel/filter paper sandwich was made and air bubbles removed by gentle pressure. This sandwich was then placed in a semi-dry electroblotting system (Transblot SD- Biorad). The electroblotter was run at 15 V for 30 minutes allowing protein from the gel to be transferred to the membrane.

The non-specific binding sites of the membrane were blocked by washing overnight in 10% low fat dried milk (Marvel-Premier beverages, Stafford, UK) diluted in 0.1% Tween in Tris buffered saline (TBS-T) on an orbital shaker at room temperature. The membrane was washed in TBS-T once for 15 minutes and twice for 5 minutes on the orbital shaker at room temperature. Primary antibody was delivered to the membrane in 5% Marvel in TBS-T and incubated at room temperature for 1 hour with gentle shaking. The primary antibodies used in this work were generally used at a

concentration between 1:2000 and 1:80. The membrane was washed in TBS-T for 30 minutes and two more washes of 15 minutes, on the orbital shaker at room temperature. The HRP labelled secondary antibody used at dilutions of between 1:2000 and 1:200, was delivered in 5% Marvel in TBS-T for 1 hour also shaking at room temperature. This was followed by a final set of washes in TBS-T, one for 30 minutes and two for 15 minutes.

The membrane was placed on cling film, protein side up, and protein was detected using ECL+ PLUS reagents (Amersham Pharmacia, UK) using the instructions of the manufacturer. The membrane was placed between cling film in a film cassette and a piece of Hyperfilm-ECL was placed into the cassette and exposed for 1 to 30 seconds under dark conditions. The film was then developed using an automated processor. Longer exposures of between 2 and 30 minutes were also taken depending on the result of the first film.

2.2.12 Polymerase chain reaction (PCR)

2.2.12.1 Primer design

In primer design, certain specific parameters were followed to encourage efficient annealing and amplification. Primers used were between 18 and 25 nucleotides in length, with a G/C concentration of 50-60% where possible. Also primer-dimer formation was minimised by avoidance of complementarity between primer pairs and within the primer itself. In terms of the gene used as template in the primer design, G/C rich areas were avoided due to secondary structure. Wherever possible, primer pairs with similar melting temperatures were used to ensure similar annealing temperatures for both primers.

2.2.12.2 Master mixes

Master mixes were mainly prepared in bulk in a designated “PCR clean area” within the department. Precautions were taken to prevent contamination of the reactions or the surrounding area, which included the use of protective clothing, filter pipette tips, gloves and sterile tubes. PCR of p35 and p40 fragments for flexi-IL-12 used the

commercial kit *Pfu* polymerase isolated from *Pyrococcus furiosus* (Promega, USA). This kit contained master mixes that were prepared according to the instructions of the manufacturer, aliquoted into 0.2 ml PCR tubes (Perkin Elmer, USA) and stored at -20°C . These master mixes consisted of optimised proportions of *Pfu* DNA polymerase enzyme, enzyme buffer, dNTPs and nuclease free dH_2O . This optimisation is to achieve a balance between specificity and quantity of product amplified. DNA template and primer pairs were added to the master mix in the "PCR clean area" and the reactions were carried out using GeneAmp 9600 cycler (Perkin Elmer, USA). Specific reaction amounts and cycling conditions are described in more detail in the relevant chapters.

2.2.13 Reverse Transcriptase- PCR

RT-PCR was carried out using total RNA prepared from MYA-1 cells using the Gentra Purescript RNA isolation kit according to the manufacturer's instructions. The Access RT-PCR system (Promega, USA) is a one-tube, two-enzyme kit which uses Avian Myeloblastosis Virus (AMV) reverse transcriptase for first strand DNA synthesis, and thermostable *Tfl* DNA polymerase from *Thermus flavus* for second strand cDNA synthesis and DNA amplification. Master mixes for this system were prepared as for PCR described above. In addition, all plasticware was RNase free and DEPC treated water was used.

Reaction mixes comprised: 0.2 mM dNTP mix, 1 μM of both upstream and downstream primer, 1 mM MgSO_4 , 0.1 $\mu\text{g}/\mu\text{l}$ AMV Reverse Transcriptase, 0.1 $\mu\text{g}/\mu\text{l}$ *Tfl* DNA Polymerase, and 20% total volume with 1 x AMV/*Tfl* Reaction Buffer. 1 pg – 1 μg total RNA was used and the reaction was made up to 50 μl with Nuclease - Free Water.

The cycling conditions used were as follows: First Strand cDNA Synthesis: Reverse Transcription step; 48°C for 45 minutes, AMV RT inactivation and RNA/cDNA/primer denaturation; 94°C for 5 minutes. Second Strand cDNA Synthesis and PCR amplification: Denaturation; 94°C for 30 seconds, Annealing; 45°C for 30 seconds, Extension; 68°C for 1 minute. 40 cycles of this was completed. The final

extension was 72°C for 10 minutes and the soaking step was 1 cycle at 4°C. In order to establish the most suitable annealing temperature, reactions were often set up with a temperature gradient between 37°C to 66°C.

2.2.14 Sequencing

Two methods of sequencing were undertaken in this project. Initially sequencing was performed using the Li-Cor 4000 model. This was subsequently replaced by the ABI Prism technique as this method produced more accurate, reliable data, particularly in G/C rich sequences.

2.2.14.1 Li-Cor 4000 model

This technique required the use of IRD800-labelled primers (MWG-Biotech) and the ThermoSequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia, UK). Cycle sequencing is based on the chain termination method [Sanger *et al.* 1977], with use of a thermostable DNA polymerase that allows multiple rounds of high temperature DNA synthesis.

A reaction mix of 500 ng plasmid DNA, 1 pmol of IRD800 labelled primer and 2 µl of reaction mix containing 45 mM of dGTP, dATP, dTTP and dCTP, reaction buffer and thermostable DNA polymerase was prepared in a 0.2 ml reaction tube and dH₂O was added to a volume of 8 µl. Cycling conditions used were as follows: 95°C for 5 minutes followed by 25 cycles of 95°C for 30 seconds, 50-60°C for 30 seconds and 72°C for 30 seconds. The annealing temperature was usually 2°C below the melting temperature of the primer used. Once this reaction was completed, 4 µl of formamide loading buffer provided with the kit was added to each reaction and the samples were ready to be loaded.

Gel plates were thoroughly cleaned and polished with isopropanol, and then assembled in the casting stand with spacers. The gel was prepared using 7.2 ml 10 x TBE, 25.2 g urea, and 4.8 ml Sequagel XR ultra pure concentrate (National Diagnostics) and made up to a total volume of 60 ml with dH₂O. A volume of 400 µl

of 10% APS was added and 3 ml of this mix was removed and placed in a bijoux. A 32 µl volume of 10% APS and 4 µl N'-tetramethyl-ethylenediamine (TEMED) was added and the mix was injected at the bottom of the glass plates and allowed to polymerise to form a plug. A volume of 36 µl of TEMED was added to the remaining gel mix and the gel was poured. The comb was placed in the top of the gel and the gel was allowed to polymerise for approximately 90 minutes. The gel was then inserted into the Li-Cor 4000 system with 1 x TBE added to buffer tanks. The comb was removed, the wells flushed with buffer and a pre-electrophoresis step performed focussing of the microscope and adjustment of gain controls. Reaction samples were denatured for 3 minutes at 95°C and then around 1-1.5 µl was loaded into each well. The gel was electrophoresed and sequence data was recorded, one run typically producing 800 bp of sequence. The data was analysed using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin).

2.2.14.2 ABI Prism Technique

Cycle sequencing reactions were prepared in 0.2 ml PCR tubes using a ready made BigDye Terminator RR master mix (Applied Biosystems, Warrington, UK). This consisted of dNTPs, dye terminators, magnesium chloride, buffer, AmpliTaq DNA polymerase and FS, which is a variant of *Thermus aquaticus* DNA polymerase. In general, around 500 ng of high-quality double stranded DNA template was included in the cycling reaction (200-500 ng). 3.2 pmol of forward and reverse primers were included. The reactions were made up to a total volume of 20 µl with dH₂O.

The cycle sequencing reaction was carried out under the following conditions: 25 cycles of the following; rapid thermal ramp to 96°C, 96°C for 10 seconds, rapid thermal ramp to 50°C, 50°C for 5 seconds, rapid thermal ramp to 60°C, 60°C for 4 minutes and rapid thermal ramp to 4°C and held until collection.

Reactions were purified by spin column using Performa DTR gel filtration cartridge kit (Edge Biosystems, USA). A fresh spin column was centrifuged at 750 g for 2 minutes to remove interstitial fluid. The column was removed from the wash tube and

placed in a new collection tube and the cycle sequencing reaction was applied to the centre of the binding column and centrifuged for 2 minutes at 750 g. The binding column was then removed, the DNA pellet dried for 15 minutes using a vacuum pump and the pellet was resuspended in 20 µl Hi Di Formamide (Applied Biosystems, UK). The samples were loaded onto a 96 well plate and electrophoresed on an ABI Prism 3100 Genetic Analyser. The chromatogram was studied by eye and also analysed in the GCG package as described above.

2.2.15 Eukaryotic cell culture

2.2.15.1 Maintenance of cells

All cell lines were maintained in plastic tissue flasks in an incubator at 37°C with 5% CO₂. 5 ml of medium was used in 25 cm² flasks, 20 ml of medium in 75 cm² flasks, and 35 ml used in 175 cm² flasks.

293T cell line

293T cells are highly transformed human renal epithelial cell line, which expresses SV40 large T antigen [DuBridge *et al.* 1987]. They were maintained as a monolayer in Dulbecco's MOD Eagle medium (DMEM), 400 IU/ml penicillin/streptomycin, 2 mM L-Glutamine, 10% FCS and 400 µg/ml G418 (gentamycin). Transfections for use in KG-1 bioassays were maintained in G418 free culture medium. They were split 1:8 every 3-4 days when confluent, using trypsin as described below.

FEA and FeLV-A cell line

FEA cells are fibroblasts derived from whole feline embryos [Jarrett *et al.* 1973]. FeLV-A cells are FEA cells infected with FeLV-A virus. These cells were maintained as a monolayer in Dulbecco's MOD Eagle medium (DMEM), 1 x penicillin/streptomycin, 2 mM L-Glutamine and 10% FCS. They were split 1:4 every 3-4 days when confluent, using trypsin as described below.

CHO (Lec8) cell line

These are Chinese hamster ovary epithelial cells derived from a parental CHO Pro-5 clone (ATCC). They were maintained as a monolayer in Nutrient mixture F-12 (HAM) with L-Glutamine, 1 x penicillin/streptomycin and 10% FCS. They were split 1:10 every 3-4 days when confluent, using trypsin as described below.

MYA-1 cell line

These are pathogen-free feline T lymphoblast cells derived from the peripheral blood. They were maintained in a 7×10^5 cell/ml suspension of RPMI 1640 medium with no L-Glutamine, 10% FCS, 1 x penicillin/streptomycin, 5×10^{-5} M of 2-ME and 1:50 dilution of interleukin-2 (IL-2). They were centrifuged and split into fresh medium every 3 days.

KG-1 cell line

KG-1 cells are bone marrow myoblast cells from acute myelogenous leukaemia. They were maintained in cell suspension at a density of between 1×10^6 and 2×10^6 cells/ml. They were split every 7 days into fresh medium of Iscove's Modified Dulbecco's medium, 20% FCS, 1 x penicillin/streptomycin and 2 mM L-Glutamine.

LN156 cell line

These are mononuclear cells recovered from equine lymph nodes from fresh post mortem cases. They were dispersed through gauze and resuspended in RPMI 1640 with gentamycin. The cells were layered over Histopaque 1083 (Sigma, UK) and centrifuged for 15 minutes at 1000 rpm. The cell band interface was taken off, washed in RPMI 1640, resuspended in freezing medium as described below and stored in liquid nitrogen [McMonagle *et al.* 2001]. On revival of the cells for bioassay, cells were maintained in RPMI 1640 with no glutamine, 4 mM L-Glutamine, 1 x penicillin/streptomycin, 10% horse serum, 57.2 μ M 2-ME and 20 μ g/ml gentamicin.

QN10 cell line

This monolayer cell line is derived from an AH927 feline fibroblast clone containing the provirus of Moloney murine sarcoma virus [Jarrett and Ganiere, 1996]. They were usually split 1:5 every 3-4 days using Dulbecco's MEM containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer, 10% FCS, 1% 100 mM sodium pyruvate, 1% 200 mM L-Glutamine and 400 IU/ml penicillin/streptomycin.

2.2.15.2 Seeding of cell lines

When seeding cells, the culture medium was removed and 4 ml trypsin-EDTA was added to 175 cm² flasks, 2 ml to 75 cm² flasks and 1 ml to 25 cm² flasks. The cells were incubated at 37°C until the cells had detached from the flask bottom. Two volumes of appropriate medium were added to neutralise the trypsin and the cells were centrifuged at 1000 rpm for 5 minutes. The cells were resuspended in fresh medium at the required cell density and were maintained in new flasks as before.

2.2.15.3 Counting cells

Total live cell count was performed using trypan blue which stains dead cells dark blue. 10 µl of cell suspension was added to 90 µl of trypan blue (Invitrogen, UK). 10 µl was loaded onto a haemocytometer with a cover slip added. The number of cells in each of the 4 open squares was counted under the 10 x objective and a mean of these was taken. This mean multiplied by 10⁵ represented the number of cells per ml.

2.2.15.4 Storing and reviving cells

Cells to be frozen down for future use were trypsinised and pelleted as described above. The cells were counted and resuspended at approximately 1 x 10⁷ cells/ml in freeze medium containing 90% FCS and 10% DMSO. This was aliquoted into 2 ml cryogenic vials and placed at -70°C overnight. The following day the tubes were placed in liquid nitrogen until future use.

On revival, cells were quickly thawed using a water bath at 37°C. Fresh culture medium was added to the cells and centrifuged for 5 minutes at 1000 rpm. The cells were resuspended in medium, transferred to a flask and maintained as before.

2.2.15.5 Transfection of cells

Cells were transfected 24 hours after seeding when they were approximately 40% confluent. Transfections were performed using LipofectAMINE Reagent (Invitrogen Life Technologies, UK) according to the instructions of the manufacturer. 3 µg DNA was used for 25 cm² flasks, 10 µg for 75 cm² flasks and 20 µg for 175 cm² flasks. In order to monitor the efficiency of the procedure, a transfection using pEGFP-C1 plasmid DNA was carried out alongside the other transfections. This was viewed using the ultraviolet camera (Photometrics GmbH Sensys, A2S / Leica system (Munich, Germany) and the proportion of cells displaying fluorescence was estimated. Following the transfection procedure, cells were incubated at 37°C with 5% CO₂ in their appropriate medium for 48 hours. Cells undergoing immunofluorescence were harvested using trypsin. Those used to demonstrate protein expression were removed from the flask using a cell scraper and lysed using either cell lysis buffer or a freeze/thaw protocol. These are explained further in the relevant chapters.

CHAPTER 3: INTERLEUKIN 12

3 INTERLEUKIN-12

3.1 INTRODUCTION

3.1.1 Structure of IL-12

Interleukin-12 (IL-12), formerly known as natural killer cell stimulatory factor and cytotoxic lymphocyte maturation factor, is a heterodimeric cytokine consisting of two subunits, p40 and p35, linked by a disulphide bond. Human IL-12 was first isolated from a B cell line RPMI 8866 [Kobayashi *et al.* 1989]. The cDNA of each subunit of IL-12 was cloned and cotransfection of vectors containing each subunit was found to yield a 70-90kDa biologically active molecule [Wolf *et al.* 1991]. The subunit p40 is 306 amino acids (aa) in size with 10 cysteine residues and four potential N-linked glycosylation sites. The p35 subunit consists of 197 aa with 7 cysteine residues and 3 potential N-linked glycosylation sites [Gubler *et al.* 1991; Wolf *et al.* 1991; Podlaski *et al.* 1992]. These subunits are encoded by distinct mRNA products [Gubler *et al.* 1991] and it was found using rodent-human hybrids that the genes were located on separate chromosomes, p40 located at 5q31-q33 and p35 found at 3p12-3q13.2 [Sieburth *et al.* 1992]. Similarly, the p35 and p40 subunits of murine IL-12 have been located at chromosomes 3 and 11 respectively [Tone *et al.* 1996]. Coexpression of both subunits is required for secretion of a bioactive IL-12 molecule [Gubler *et al.* 1991].

Feline IL-12 was fully sequenced by Schijns *et al.* [1997], and Hanlon [1999], by reverse transcription of mRNA extracted from lipopolysaccharide (LPS) stimulated PBMCs. The p35 subunit consisted of 685 bp and shared 91%, 82%, 85% and 55% identity with the canine, bovine, human and murine sequence respectively at amino acid level. The p40 subunit was 1006 bp and shared 92%, 85%, 84% and 68% identity respectively, also at amino acid level. The full-length cDNA was subsequently cloned into separate plasmids and bioactivity confirmed using CTL enhancement by human PBMCs [Imamura *et al.* 2000].

IL-12 p35 and p40 subunits have no sequence homology, nor are they structurally related. The p35 subunit is 35kDa in size and its structure, as in the case of most cytokines, is alpha helix rich. In terms of sequence, p35 shows homology with IL-6 and granulocyte-colony stimulating factor (G-CSF) [Merberg *et al.* 1992]. In contrast, the 40kDa p40 subunit shows no homology with other cytokines and is related to the haemopoietin family of receptors. Of this group, p40 correlates most with the extracellular domain of the α -subunit of IL-6 receptor and the ciliary neurotrophic factor receptor [Gearing and Cosman, 1991; Schoenhaut *et al.* 1992].

3.1.2 Antagonism of p40 homodimer to heterodimeric IL-12

Production of p40 subunit has been shown to result not only in p40 monomer, but also a disulphide-linked p40 homodimer [Gillesen *et al.* 1995]. It has been demonstrated that lymphoblastoid cell lines produce in the region of 5-500 fold excess p40 compared to heterodimeric IL-12 [D'Andrea *et al.* 1992; Podlaski *et al.* 1992]. Of circulating p40, about 20-40% consists of p40 homodimer in endotoxaemic murine serum [Heinzel *et al.* 1997].

Supernatant from p40 transfections have been shown to antagonise IL-12 heterodimer, inhibiting proliferation and IFN γ production by mouse splenocytes and inhibition of the effect of IL-12 on differentiated T helper 1 cells [Mattner *et al.* 1993]. The p40 homodimer is 25-50 fold more effective than the monomer at producing specific inhibition of IL-12 function [Gillesen *et al.* 1995]. Work has shown that this antagonism is due to the ability of the homodimer to bind the IL-12 receptor without eliciting biological activity [Ling *et al.* 1995]. It is therefore important that this potential for antagonism is addressed in the design of constructs encoding IL-12.

Some work has suggested that p40 alone may play a role in some immune responses. Allografted p35 deficient mice were shown to have higher Th1 responses than p40 deficient mice. Th1 enhancement was then suppressed by treatment with anti-p40 monoclonal antibody [Piccotti *et al.* 1998].

3.1.3 IL-12 receptor

Both human and murine IL-12 receptor cDNA has been cloned and the receptor consists of two subunits, IL-12R β 1 and IL-12R β 2 [Chua *et al.* 1994; Presky *et al.* 1996]. IL-12R β 1 subunit is a 662 aa (100kDa) type 1 transmembrane protein, which has a cytoplasmic domain of 91 aa and an extracellular domain of 516 aa. IL-12R β 2 subunit is also a type 1 transmembrane protein of 862 aa (130kDa) with cytoplasmic and extracellular domains of 216 and 595 aa respectively. Both are members of the cytokine receptor superfamily and have closest homology to gp130 β -type cytokine receptor, leukaemia-inhibitory factor and granulocyte-colony-stimulating factor receptors. [Chua *et al.* 1994; Presky *et al.* 1996].

Recombinant IL-12R β 1 and IL-12R β 2 exist as disulphide-linked dimers or oligomers on the cell surface. When these subunits are expressed individually, they each bind IL-12 with only low affinity. High affinity binding occurs with coexpression of both subunits [Chua *et al.* 1994; Presky *et al.* 1996]. The murine IL-12 receptor subunits show similar structural homology to their human counterparts. Differences however have been found in the formation of high and low affinity receptors for murine IL-12 [Chua *et al.* 1994; Presky *et al.* 1996]. In both species however, it appears that the IL-12 β 2 subunit acts as the signal-transducing element of the high affinity receptor formation [Presky *et al.* 1996; Zou *et al.* 1997].

The IL-12 receptor is expressed mainly on Th1 cells such as T and NK cells [Desai *et al.* 1992]. The receptor may be found on resting NK cells but only on activated T cells such as those treated with mitogens or alloantigens to produce receptor upregulation [Desai *et al.* 1992]. IL-2 and IL-12 can also activate NK cells bringing about upregulation of IL-12 receptor and IL-4 and IL-12 act synergistically to upregulate receptor expression [Desai *et al.* 1992; Naume *et al.* 1993]. The fact that expression of IL-12R β 2 subunit is mainly restricted to Th1 cells has led to the suggestion that it is this subunit that brings about sensitivity to IL-12 [Rogge *et al.* 1997; Szabo *et al.* 1997]. These studies also showed that Th2 cells express IL-12R β 1 subunit but not IL-12R β 2 and it has been suggested that upregulation of this

subunit on Th2 cells may play a role other than formation of the high affinity IL-12 receptor complex [Gately *et al.* 1998].

In terms of signal transduction, it has been established that the IL-12 receptor brings about activation of signal transducers and activators of transcription 3 (STAT 3) and STAT 4 [Jacobson *et al.* 1995]. Evidence suggests that IL-12R β 1 interacts with Janus-family kinase 2 (JAK2) whereas IL-12R β 2 uses TYK2, STAT3 and STAT4 in signal transduction [Lamont and Adorini, 1996]. As will be explained later, this signal transduction mechanism is distinct from that of IL-18 and it is activation of separate pathways that explains the synergistic effect of these cytokines on target cells.

3.1.4 Production of IL-12

Human IL-12 was first isolated from an EBV-transformed human B cell line RPMI 8866 [Kobayashi *et al.* 1989]. This cytokine is produced mainly by monocytes, macrophages and B cells [D'Andrea *et al.* 1992]. PBMCs also produce IL-12 in response to various stimulatory factors such as LPS, *Staphylococcus aureus* and *Mycobacterium tuberculosis* [D'Andrea *et al.* 1992; Baron *et al.* 1993]. It is thought that IL-12 production is brought about by interaction of the CD40 ligand (CD40L) of activated T cells with CD40 present on cells such as monocytes [Shu *et al.* 1995].

Other cells have also been shown to exhibit low levels of IL-12 production such as Langerhans cells, follicular dendritic cells [Trinchieri, 1994] and also LPS and IFN γ -stimulated granulocytes [Cassatella *et al.* 1995]. Various studies have demonstrated the *in vivo* production of IL-12 by human keratinocytes when contact allergen was applied to the skin, implying a possible role of IL-12 in dermal immune responses [Muller *et al.* 1994]. In other studies, these cells demonstrated constitutive p40 mRNA expression while requiring phorbol ester stimulation to induce p35 mRNA expression [Aragane *et al.* 1994].

3.1.5 Regulation of IL-12 production

3.1.5.1 Stimulation of production

Three major factors are thought to be involved in the upregulation of IL-12 production (figure 3.1). These are 1) cytokines such as IFN γ , 2) induction of IL-12 by bacteria and certain microorganisms, 3) costimulation of IL-12 by CD40/CD40L interaction [M^cDyer *et al.* 1998].

1) It has been demonstrated that pretreatment of monocytes with IFN γ primes the cells, stimulating enhancement of p35 and p40 mRNA expression on exposure to LPS [Hayes *et al.* 1995]. In addition a study has suggested that mycobacteria-induced p40 induction from bone marrow-derived macrophages depends on the presence of tumour necrosis factor- α (TNF- α) and IFN γ [Flesch *et al.* 1995]. Work has shown that the stimulatory effect of IFN γ arises from its ability to enhance the expression of CD40 on monocytes [Alderson *et al.* 1993].

Evidence suggests, however, that the presence of IFN γ is not always essential for the stimulation of IL-12 production. IFN γ -deficient mice infected with *Toxoplasma gondii* or *Listeria monocytogenes* have been shown to produce p40 subunit after stimulation *in vitro* implying that there are other stimulatory factors involved in IL-12 expression [Scharton-Kersten *et al.* 1996; Szalay *et al.* 1996].

Although IFN γ is recognised as the major cytokine in upregulation of IL-12, other molecules such as granulocyte macrophage-colony stimulating factor (GM-CSF) have also been found to have a role [Kubin *et al.* 1994].

2) Infection of cells with *T. gondii* can induce the production of IL-12 in the absence of both IFN γ and CD40L [Sousa *et al.* 1997]. This demonstrates an alternative pathway by which IL-12 may be stimulated, where APCs directly produce IL-12 in response to exposure to antigenic material.

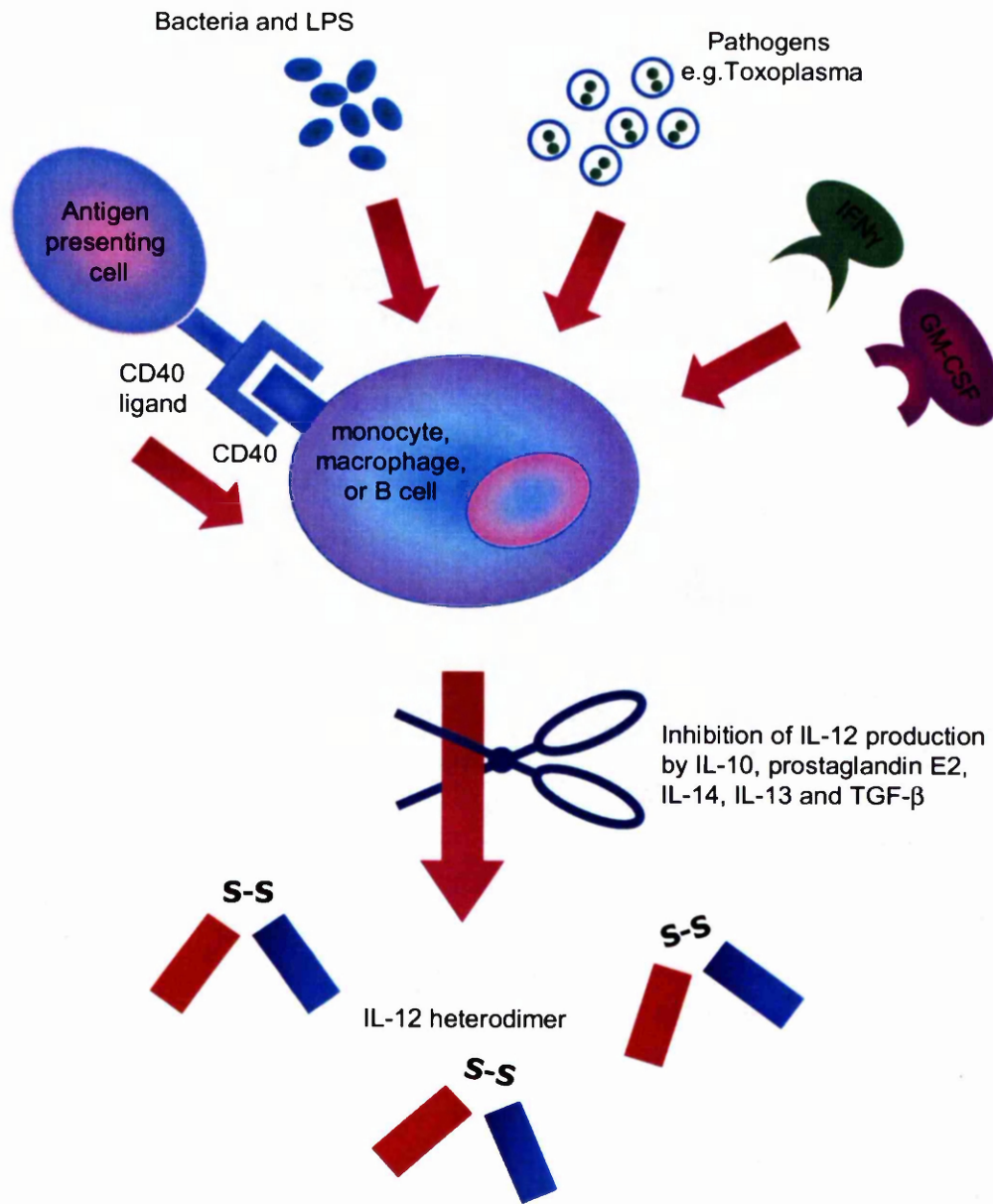
3) As mentioned previously, T cell activation stimulates CD40L expression, which on interaction with CD40 present on APCs induces IL-12 production [Shu *et al.* 1995]. IL-12 production by PBMCs stimulated with phytohaemagglutinin (PHA) was completely suppressed by anti-CD40L antibody implying that in this case, CD40/CD40L interaction was vital for the production of IL-12 protein [McDyer *et al.* 1998].

3.1.5.2 Inhibition of IL-12 production

Various factors are known to inhibit IL-12 production. The most widely studied is IL-10, which has been shown to inhibit human lymphocyte IFN γ production by suppressing the synthesis of IL-12 in accessory cells [D'Andrea *et al.* 1993].

Prostaglandin E2 has also been shown to have an inhibitory effect on IL-12 production in LPS-stimulated human peripheral blood cells [van der Pouw Kraan *et al.* 1995], as has IL-4, IL-13 and tumour growth factor- β (TGF- β) [D'Andrea *et al.* 1995]. Certain pathogens have also shown an ability to suppress the production of IL-12. For example, exposure of *S. aureus* or IFN γ -stimulated monocytes to measles virus reduced IL-12 protein production, and cross-linkage with the CD46 measles receptor produced the same effect [Karp *et al.* 1996].

Figure 3-1 Stimulation and inhibition of IL-12



3.1.6 Biological activities of IL-12

Interleukin-12 has been shown to exhibit several biological functions that make this cytokine crucial in the immune response to pathogens. A diagram showing the functions of this cytokine is shown at the end of this section (figure 3.2). Firstly IL-12 activates and stimulates the proliferation of T and NK cells which contribute to the first line of defence against pathogenic microorganisms. In addition, IL-12 has been shown to encourage the development of T helper type 1 responses and also the differentiation of CTLs. IL-12 therefore promotes cell-mediated immune responses to infection and is particularly important in combating intracellular organisms such as viruses.

3.1.6.1 Cytotoxicity of NK cells

IL-12 has been shown to enhance the *in vitro* cytotoxicity of T cells [Kobayashi *et al.* 1989] and NK cells [Robertson *et al.* 1992]. Increased lysis of HIV-infected target cells and antibody-coated target cells was shown by NK cells treated with IL-12 [Chehimi *et al.* 1993]. This effect of IL-12 corresponded to enhanced binding and increased granularity of the NK cells [Cesano *et al.* 1993; Chehimi *et al.* 1993], which was associated with an upregulation of a variety of cell adhesion molecules and activation markers such as CD2, CD11a, CD25, CD56, CD54, CD69, CD71 [Rabinowich *et al.* 1993]. IL-12 also increases the frequency of NK cell interaction with its target cells by enhancing the migratory activity [Rabinowich *et al.* 1993], and stimulating chemotaxis of NK cells [Allavena *et al.* 1994]. The increase in the granular nature of NK cells after IL-12 treatment is due to increased protein and mRNA expression of cytotoxicity-related proteins, enhancing the cytolytic ability of these cells still further [Aste-Amezaga *et al.* 1994].

When the optimal cytotoxic effect of IL-12 is compared with other cytokines with the same ability such as IFN γ and IL-2, IL-12 produces a lower response in NK cells. However, the lowest concentration required to increase cytotoxicity was found to be 3-fold higher for IFN γ and IL-2 than for IL-12, suggesting that NK cells may be more sensitive to IL-12 [Kobayashi *et al.* 1989].

IL-12 may play a role in the formation of lymphokine activated killer (LAK) cells. These cells arise when lymphocytes are cultured *in vitro* with cytokines such as IL-2 and exhibit MHC-unrestricted killing properties. It is unclear whether these cells also occur *in vivo*, or whether they are a result of exposure to high cytokine concentrations *in vitro* [Male *et al.* 1996a]. It has been found that the formation of LAK cells by IL-2 was decreased when incubated with an anti-IL-12 antibody, implying that IL-12 may be involved in the generation of these cells [Gately *et al.* 1992].

3.1.6.2 Cytotoxicity of T cells

IL-12 also increases the cytotoxicity mediated by T cells. Peripheral blood T cells were found to lyse Fc-receptor-positive target cells coated with anti-CD3 antibody after treatment with IL-12 [Chehimi *et al.* 1993]. IL-12 has also been shown to bring about a 10-20 fold increase in CTL production from CD8⁺ cells specific for anti-CD3 hybridoma target cells [Mehrotra *et al.* 1993]. Studies suggest that IL-12 enhances both the generation and activity of CTLs as the increased generation of allospecific murine CTL from leukocyte cultures was comparable when IL-12 was used at the beginning or the end of leukocyte incubation [Bloom *et al.* 1994].

3.1.6.3 T and NK cell proliferation

In order to induce proliferation of T and NK cells in culture, both must first be preactivated with a stimulant such as PHA [Desai *et al.* 1992]. Upon activation, IL-12 has been found to induce the proliferation of both T and NK cells [Wolf *et al.* 1991]. This mitogenic effect is observed in preactivated CD4⁺ and CD8⁺ T and NK lymphoblasts, but in NK and T lymphoblasts with T cell receptor (TCR)-gamma delta⁺, IL-12 can significantly inhibit proliferation [Perussia *et al.* 1992]. This is thought to be due to IL-12-dependent TNF production, as treatment of cells with antibody to TNF re-established 70% of the inhibited proliferation. This proliferative effect has been found to coincide with IL-12 receptor expression of these cells [Desai *et al.* 1992].

It has also been demonstrated that IL-12 alone does not induce proliferation of PBMCs, but on pretreatment with low doses of IL-2, enhancement of proliferation is

observed [Gately *et al.* 1991]. This is a consequence of IL-2-induced IL-12 receptor expression stimulated by IL-2 [Desai *et al.* 1992].

Both human CTL and T helper type 1 (Th1) cells have been shown to proliferate in response to IL-12 under certain parameters. CTLs require preactivation by anti-CD3 antibodies to induce proliferation [Bertagnolli *et al.* 1992], whereas Th1 cells required stimulation with antigen, anti-CD3 antibodies or mitogens [Trinchieri *et al.* 1994].

In general, the presence of IL-2 is not required to produce the proliferative effect by IL-12 [Gately *et al.* 1991]. In addition, IL-4, CD28-ligand and B7.1 molecules can also under certain circumstances, augment IL-12-mediated proliferation. IL-4 and IL-12 have a synergistic effect on CD56⁺ NK cell proliferation [Naume *et al.* 1993]. IL-12 and B7/CD28 interaction produces a strong synergistic effect in stimulating cytokine production and proliferation of peripheral blood T cells [Kubin *et al.* 1994].

3.1.6.4 Regulation of Th1 and Th2 cells

Naive CD4⁺ T cells have the ability to differentiate into either Th1 or Th2 cells. Th1 cells promote cell-mediated immunity by the production of IL-2 and IFN γ . Conversely, Th2 cells encourage humoral immunity by the production of IL-4, IL-5, IL-6, IL-10 and IL-13 [Scott *et al.* 1993; Paul and Seder, 1994]. An important function of IL-12 is its ability to promote the *in vitro* production of T helper 1 responses, which have been shown in both humans [Manetti *et al.* 1993] and mice [Hsieh *et al.* 1993]. This has also been demonstrated *in vivo* [Magram *et al.* 1996], where IL-12 deficient mice displayed impaired Th1 responses measured by the IFN γ response to endotoxin administration.

IL-12 influences Th1 cell development and function in several ways. Firstly it is required for the differentiation of naive T cells into Th1 cells on activation by an antigen [Schmitt *et al.* 1994]. IL-12 also acts as a costimulant in inducing the production of IFN γ from stimulated Th1 cells treated with B7 [Murphy *et al.* 1994]. Finally, memory T cells previously exposed to antigen can be stimulated to

differentiate into IFN γ -producing Th1 cells by incubation with IL-12 [Marshall *et al.* 1995].

The IL-12 related promotion of Th1 immune responses are inhibited in some cases by anti-IFN γ antibody [Schmitt *et al.* 1994]. However, some Th1 responses could not be inhibited in this way. For example the effect of IL-12 on cytokine secretion by Dermatophagoides pteronyssinus group 1 (Der p 1)-specific T cells was not prevented by the use of anti-IFN γ [Manetti *et al.* 1993]. IFN γ is incapable of inducing Th1 cell development alone, instead acting as a costimulator of IL-12 as previously described [Wenner *et al.* 1996]. Also, the Th1 response induced by IL-12 does not stimulate the production of the other major Th1 related cytokine, IL-2 [Magram *et al.* 1996].

IL-12 also inhibits the production of IL-4 from antigen specific T cell lines. IL-4 can induce the development of Th2 cells from CD4⁺ T cells and is the dominant influence over CD4⁺ cells when both IL-12 and IL-4 are present [Hsieh *et al.* 1993]. Incubation of T cells specific for Der p 1 with IL-12 demonstrated increased IFN γ stimulation and inhibition of IL-4 production. This cytokine profile represents a predominance of Th1 rather than Th2 clones [Manetti *et al.* 1993]. This experimental system was further used to show that IL-12 probably increases Th1 responses by priming clone progenitors to differentiate into Th1 cells rather than selecting precommitted T cell precursors [Manetti *et al.* 1994].

The bias of IL-12 towards a Th1 type response however is not ubiquitous, and under certain conditions IL-12 may produce Th2 responses in cells. For example, in IFN γ knockout mice, IL-12 induced Th2-dependent granuloma formation and increased IgE levels. The induction of IFN γ by IL-12 therefore appears to be responsible for the IL-12 induced suppression of Th2 responses [Wynn *et al.* 1995].

3.1.6.5 Cytokine production and regulation by IL-12

Although IFN γ is the main cytokine induced by IL-12, several others may be induced in smaller amounts such as TNF α , IL-2 [Perussia *et al.* 1992], GM-CSF, [Aste-Amezaga *et al.* 1994] and IL-10 [Gerosa *et al.* 1996]. This is interesting as IL-10 is a

cytokine that stimulates Th2 responses. IL-10 is able to inhibit both the synthesis and action of IL-12, possibly acting as a negative feedback mechanism on the cytokine [D'Andrea *et al.* 1993; Tripp *et al.* 1993]. IL-12 has also been shown to inhibit the production of transforming growth factor β (TGF β) [Marth *et al.* 1997].

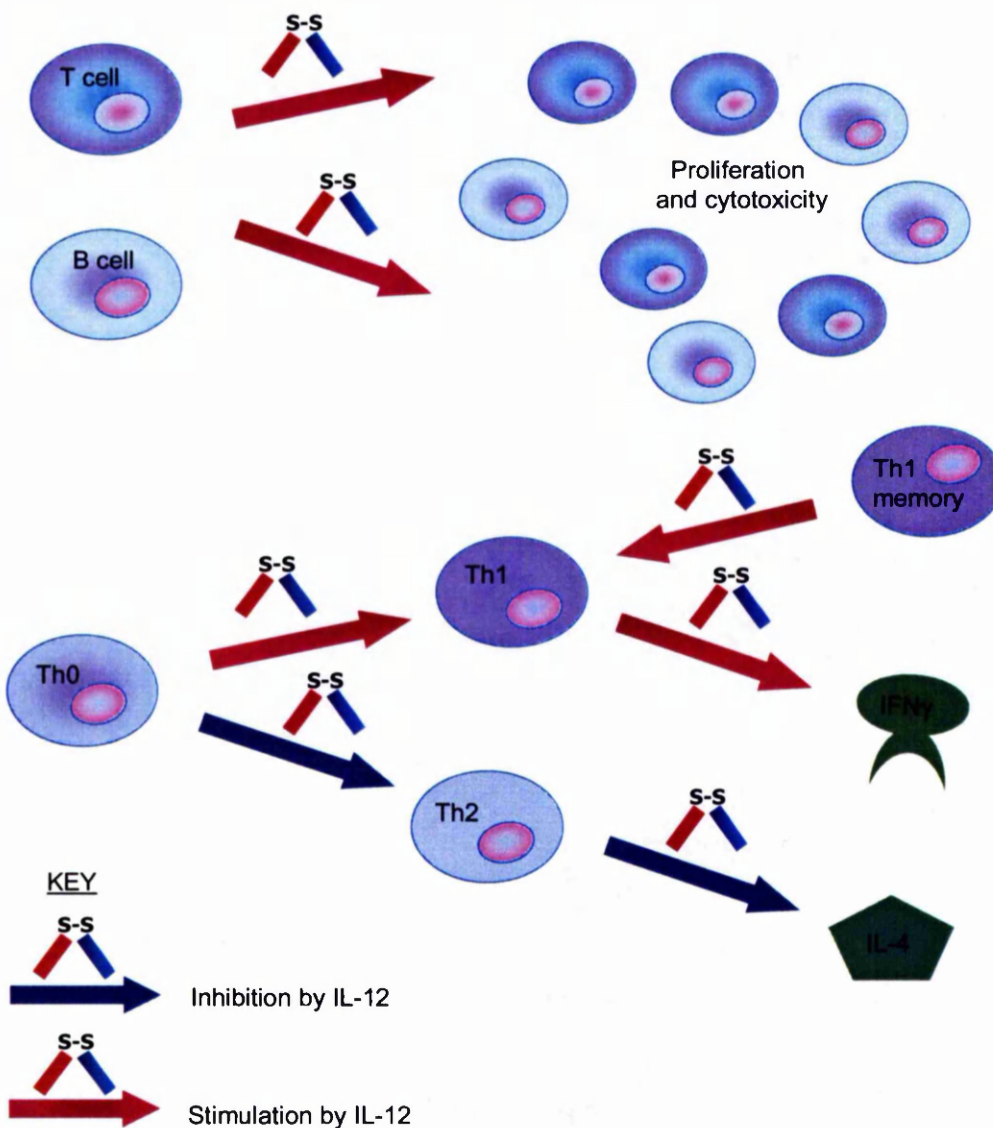
IL-12 can function in synergy with several other cytokines in the production of IFN γ such as TNF α [Aste-Amezaga *et al.* 1994], IL-2 [Tripp *et al.* 1993], and IL-1 [Wu *et al.* 1993]. Conversely IL-12 related IFN γ production can be inhibited in the presence of other cytokines such as IL-10 [Tripp *et al.* 1993], TGF β [Hunter *et al.* 1995] and IL-4 [Kiniwa *et al.* 1992].

3.1.6.6 Effect of IL-12 on humoral immunity

In mice, Th1 type antibody responses tend to be associated with the secretion of IgG2a, IgG2b and IgG3 antibodies [Finkelman *et al.* 1990]. IL-12 has been shown to upregulate these antibody isotypes in mice *in vivo* [Germann *et al.* 1995], and suppress Th2 associated IgG1 responses after primary IL-12 immunisation [McKnight *et al.* 1994; Buchanan *et al.* 1995]. Successive exposure to IL-12 however has been shown to enhance IgG1 levels [Buchanan *et al.* 1995] and in this study, treatment with anti-IFN γ antibody eliminated the initial IgG1 suppression but had no effect on the increased IgG2a levels demonstrated. This suggests that the antibody responses stimulated by IL-12 are influenced by the IL-12-induced IFN γ , although other factors or cytokines are probably also involved.

IL-12 has also been shown to influence IgE antibody responses. The *in vitro* synthesis of IgE is suppressed in IL-4 treated lymphocytes by IL-12 [Kiniwa *et al.* 1992], whereas in some cases IL-12 may significantly enhance IgE secretion [Wynn *et al.* 1995]. It is likely in this case that IgE is produced in response to IL-4 stimulation by IL-12, which takes place under certain experimental circumstances as described previously.

Figure 3-2 Biological functions of IL-12



3.1.7 Potential clinical role of IL-12

The important role that IL-12 plays in the immune response has led to investigation into its potential therapeutic value. Studies have demonstrated the use of IL-12 in the treatment of infectious disease such as *Toxoplasma gondii* [Khan *et al.* 1994], *Mycobacterium tuberculosis* [Cooper *et al.* 1995], *Leishmania major* [Heinzel *et al.* 1991; 1993], and also tumours [Brunda *et al.* 1993; Rakhmilevich *et al.* 1996]. IL-12

has been shown to play a role in immune-related diseases and treatment to decrease IL-12 levels have been found to be beneficial. Examples of these include experimental autoimmune encephalomyelitis [Issazadeh *et al.* 1995; Gately *et al.* 1998], and collagen-induced arthritis [McIntyre *et al.* 1996]. Of direct relevance to this project is the potential use of IL-12 as an adjuvant to vaccination.

3.1.7.1 IL-12 as a vaccine adjuvant

The stimulation of Th1 responses and consequent promotion of cell mediated immunity by IL-12, has led to its use in vaccinology where cell mediated responses against the pathogen would be beneficial.

Coadministration of Th1 promoting cytokines with DNA vaccination can increase Th1 cells and CTL activity, while suppressing Th2 responses [Chow *et al.* 1998]. It is well documented that CTL activity is crucial for the clearance of intracellular pathogens, particularly viruses. Expression vectors encoding IL-12 have been used as adjuvants to DNA vaccines to human immunodeficiency virus-1 (HIV-1). The use of an IL-12 plasmid significantly increased the specific CTL response to a variety of HIV-1 DNA vaccines [Kim *et al.* 1997]. This CTL response was associated with an increase in IFN γ production, an effect that was abrogated by the use of anti-IFN γ antibody [Tsuji *et al.* 1997]. This shift towards cell-mediated immunity was in contrast to some studies that showed that IL-12 increased only the magnitude of the immune response to an HIV *env* DNA vaccine rather than the nature of the response [Moore *et al.* 2002].

This type of vaccine can also be administered by topical application involving prior removal of keratinocytes by fast-acting adhesive [Liu *et al.* 2002]. In this case, coadministration of IL-12 vector resulted in enhancement of CTL response and the delayed-type hypersensitivity response (DTH).

The adjuvant effect of IL-12 on DNA vaccines has been studied using a variety of other viruses. One study investigated the immunisation of mice with a DNA vaccine to hepatitis B virus with IL-12 or IFN γ genes. DNA vaccination corresponded with increased Th1 cells, CTL activity and IgG2a antibody production, with inhibition of

Th2 cells and IgG1 antibody. In contrast, coadministration with the Th2 cytokine IL-4 stimulated the opposite effect [Chow *et al.* 1998]. In addition, coinjection of an IL-12 vector with a herpes simplex virus-2 vaccine, decreased morbidity and mortality of mice. This was due to increased Th1 type CD4⁺ T cell responses producing an increase in IL-2 and IFN γ levels and inhibition of IL-4 and IL-10 [Sin *et al.* 1999].

IL-12 has been used as an adjuvant to vaccines for both parasitic and bacterial pathogens. BALB/c mice vaccinated with soluble leishmanial antigen alone or IL-12 plasmid alone failed to protect mice from disease. However coadministration produced complete protection and long-term immunity along with enhanced antigen-specific IFN γ production. This effect was abrogated by the use of anti-IL-12 or anti-IFN γ antibody [Yamakami *et al.* 2001]. Also, coimmunisation of a DNA vaccine to *Mycobacterium tuberculosis* with IL-12 demonstrated increased protection from challenge and increased both the specific lymphocyte proliferative response and level of IFN γ producing T cells [Palendira *et al.* 2002].

In several studies, IL-12 had a negative adjuvant effect on DNA vaccination. IL-12-expressing plasmid suppressed the protective immunity of a DNA vaccine to Japanese encephalitis virus. This was associated with reduced specific T cell proliferation and antibody responses and the effect was dependent on endogenous IFN γ production [Chen *et al.* 2001]. Finally, adverse effects of a feline IL-12 plasmid were demonstrated in DNA vaccination against feline infectious peritonitis virus (FIPV). Coinoculation in this case enhanced susceptibility to challenge with the virus [Glansbeek *et al.* 2002].

3.1.7.2 The role of IL-12 as an adjuvant to feline retrovirus DNA vaccines

DNA vaccination against feline retroviruses has demonstrated some success when used in conjunction with IL-12 plasmid vectors. A vaccine for FeLV coinjected with IL-12 and IL-18 expression vectors elicited complete protection against both transient and persistent viraemia and protected five of six kittens from latent viraemia [Hanlon *et al.* 2001]. These protected cats were found to have higher levels of virus-specific

CTLs in peripheral blood and lymphoid organs than unprotected control cats [Flynn *et al.* 2000a].

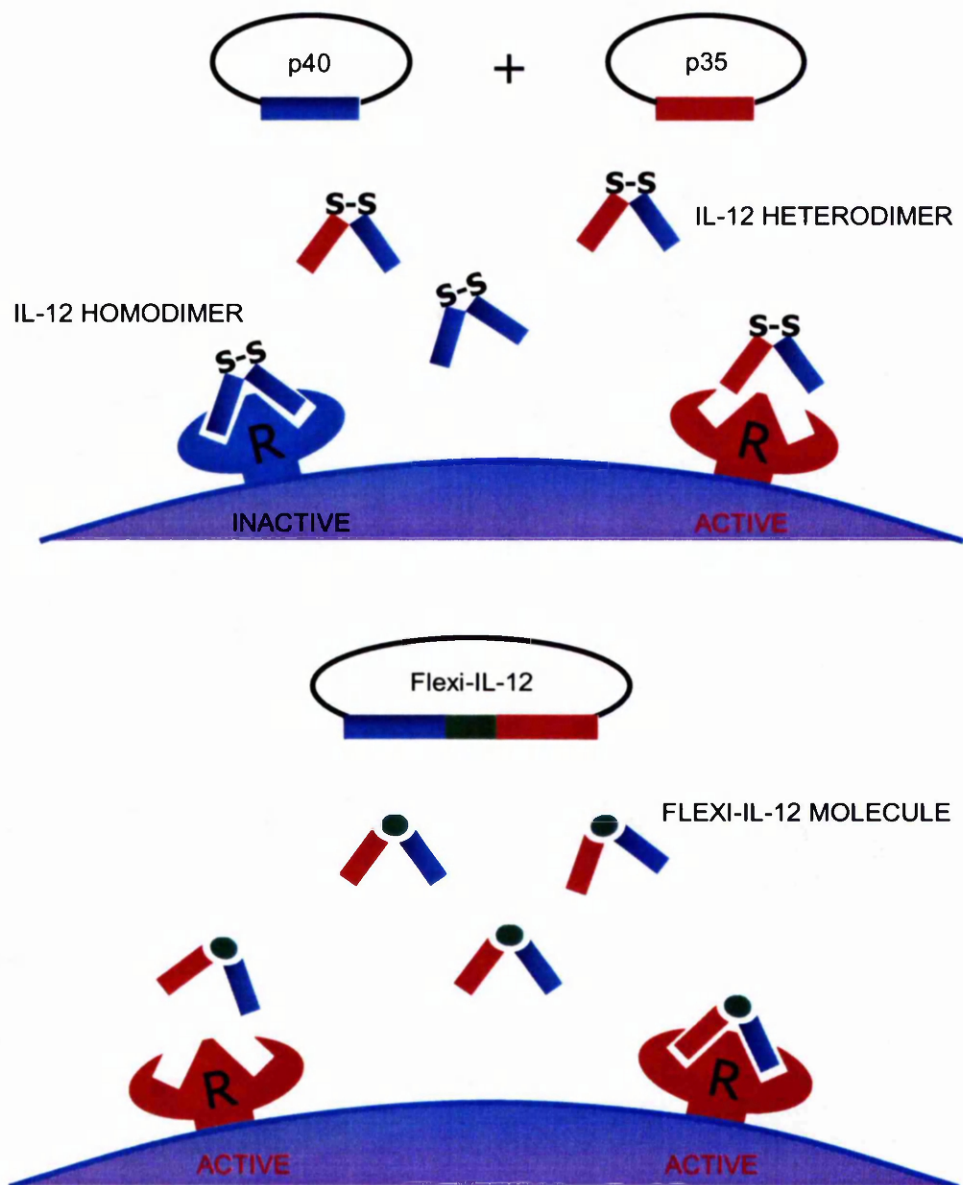
In contrast, when IL-12 vector was used in conjunction with DNA vaccines to feline immunodeficiency virus (FIV) and IL-18 vector, this had either no effect on protection against reverse transcriptase defective FIV cDNA and actually decreased protection induced by an integrase defective FIV cDNA. When virus-specific CTL responses were measured, IL-12 elicited no enhancement of the former vaccine and a decrease in CTL level for the latter vaccine [Dunham *et al.* 2002]. However in a different study, a DNA vaccine to FIV gp140 protein in combination with IL-12 expression vector demonstrated significant immune protection [Leutenegger *et al.* 2000].

3.1.8 Structural conformation of IL-12 constructs

The heterodimeric structure of IL-12 has presented some problems in the development of IL-12 plasmid constructs. As mentioned previously, p40 monomer has been shown to form a disulphide-linked homodimer, which is able to bind the IL-12 receptor without evoking biological activity [Ling *et al.* 1995]. The homodimer therefore acts as a physiological antagonist to IL-12 heterodimer by suppression of the biological effect of the functional molecule [Mattner *et al.* 1993]. It is therefore important that the production of p40 homodimer is minimised in the construction of a vector for IL-12.

IL-12 DNA may be administered by the inoculation of separate plasmid vectors encoding p35 and p40 subunits [Hanlon *et al.* 2001]. The potential disadvantages of this are that it relies on the transfection of each host cell with both plasmids in order to produce IL-12 protein. In addition, there is potential for excess production of p40 which theoretically may lead to antagonism problems (figure 3.3 top).

Figure 3-3 Comparison of separate IL-12 subunit plasmids with flexi-IL-12



Several IL-12 constructs have since been designed in an attempt to overcome these problems and are listed as follows:

- 1) A single plasmid vector containing both p35 and p40 subunit DNA in tandem either under the same promoter [Yamakami *et al.* 2001], or separate promoters [Tsuji *et al.* 1997].
- 2) An internal ribosome entry site sequence (IRES) of encephalomyocarditis virus between the coding sequences of each subunit [Zitvogel *et al.* 1994].

- 3) IL-12 subunits linked by cDNA encoding the foot and mouth disease virus self-cleaving peptide, 2A. This sequence produces protease cleavage of the polypeptide into separate subunits on translation [Kokuho *et al.* 1999].
- 4) Fusion of p35 and p40 subunits with a 45 base pair synthetic linker sequence to produce a single chain fusion construct [Anderson *et al.* 1997].

The single plasmid expressing both subunits in tandem still has the potential for p40 overexpression and IL-12 antagonism. In plasmids where the subunits are under the control of a single promoter, the subunit downstream in these constructs has a tendency to be expressed at a lower level. Subunits controlled by separate identical promoters are more likely to express similar levels of subunit. Although the IRES construct increases expression of the subunit downstream, there is still a tendency for the 3' protein to be expressed at a lower level and equimolar concentrations of p35 and p40 are not guaranteed. Both IL-12 protease cleavage construct and the single chain fusion construct guarantee comparable protein expression of each subunit, although the cleavage construct allows production of free p40 which could form p40 homodimer.

The single chain fusion construct uses a synthetic polypeptide linker based on a system used previously to construct single chain antibodies [Huston *et al.* 1993]. The linker consists of a chain of Gly-Gly-Gly-Gly-Ser repeated three times followed by the subunit downstream minus its leader sequence [Anderson *et al.* 1997]. On translation, this fusion protein mimics the biological functions of recombinant IL-12 (figure 3.3 bottom) such as activated T cell proliferation, production of IFN γ from activated T cells, activation of NK cells and phosphorylation of the same intracellular signal transduction proteins as rIL-12 [Anderson *et al.* 1997]. An equine form of IL-12 fusion protein has since been cloned and bioactivity confirmed by means of an IFN γ induction assay [McMonagle *et al.* 2001]. In addition, the use of a murine fusion protein construct *in vivo* has demonstrated protection when used in tumour immunotherapy [Lode *et al.* 1998].

The bioactivities of several IL-12 constructs were compared by cloning both murine and human constructs into a retroviral vector [Lieschke *et al.* 1997]. Separate

plasmids encoding p40 and p35 were used along with IRES and single chain fusion IL-12. In the case of the latter two constructs, subunits were arranged in both 5'-p40/p35-3' and 5'-p35/p40-3' orientations. Of all the constructs, the single chain fusion IL-12 plasmid with 5'-p40/p35-3' orientation showed the highest level of IL-12 in the bioassay and showed the highest relative specific activity. The human IL-12 fusion construct was also found to be bioactive with high specific activity. It was on the basis of these results that this fusion protein was selected for use in our work.

3.1.9 Aim of work

Given the recent development of a murine IL-12 fusion construct which expresses p40 and p35 linked by a synthetic polypeptide linker sequence [Anderson *et al.* 1997], this chapter describes the cloning of an equivalent feline fusion construct (flexi-IL-12) and demonstrates its *in vitro* expression and bioactivity. This flexi-IL-12 structure overcomes the major potential disadvantages existing in other constructs by ensuring cotransfection and equimolar levels of expression of both p35 and p40 subunits. *In vitro* expression of feline flexi-IL-12 protein was performed by Western blot analysis. *In vitro* bioactivity was demonstrated using an assay that measures the dose dependent production of equine IFN γ from equine lymph node cells when exposed to feline IL-12 protein.

3.2 MATERIALS AND METHODS

3.2.1 Cloning strategy of flexi-IL-12

Flexi-IL-12 consists of the two subunits of IL-12, p40 and p35, linked by a synthetic "flexi" linker sequence. As bioactivity of murine flexi-IL-12 was found to be maximal when p40 was situated at the 5' end of the construct [Lieschke *et al.* 1997], a similar strategy was adopted to generate feline flexi-IL-12. Thus an intact p40 subunit cDNA was linked to signal peptide deleted p35 subunit cDNA (Δ p35), through a glycine-serine rich peptide sequence [Anderson *et al.* 1997].

The cloning strategy of this construct was based on the separate PCR of each subunit, using primers that encoded 5' and 3' subunit sequence of feline IL-12 [Hanlon, 1999]. The 3' p40 primer encoded a portion of the flexi sequence with a *Nar* I restriction site at the 3' end and the 5' Δ p35 primer encoded a *Nar* I site with the 3' portion of the linker sequence. This restriction site was then used to unite the two linker sequences to produce the complete flexi-IL-12 cDNA sequence. Other restriction sites were included in the primers to allow excision of the flexi-IL-12 cDNA from the intermediate vector into the expression vector (pCI-neo, Promega). This cloning strategy is summarised in figure 3.4.

Mapping of primers

5'p40 primer

```
5'-GCGGTTAACGTCGACACGCCACGATGGATCCTCAG-3'
      < HpaI >< SalI > < KOZAK >  M   G   P   Q
```

3'p40 primer

```
5'GTGTCCTGCAGTGGTGGCGGTGGCTCGGGCGGTGGTGGATCGGGCGCCGCG-3'
      V   S   C   S<-----linker----->< NarI >
```

5'p35 primer

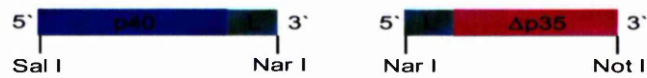
```
5'-GCGGGCGCCGGTGGCGGATCTAGGAACCTCCCCACAC-3'
      < NarI ><----linker---->   R   N   L   P
```

3'p35 primer

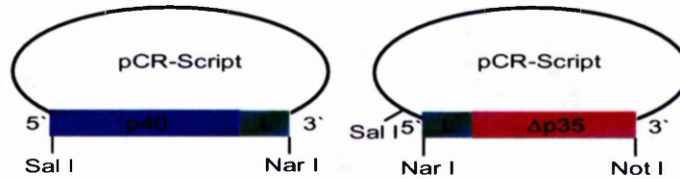
```
5'-CTGAATGCTTCCTAAGCGGCCGCGTTAACGAGCTCGC-3'
      L   N   A   S   <   NotI  >< HpaI >< SacI >
```

Figure 3-4 Diagram depicting feline flexi-IL-12 cloning strategy

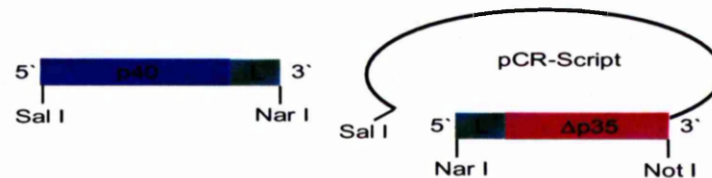
1) PCR amplification



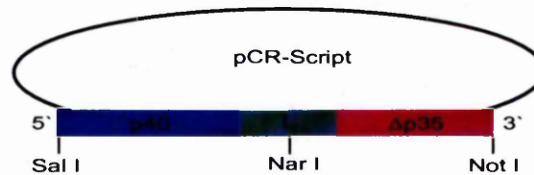
2) Ligation into pCR-Script
Sequencing of clones



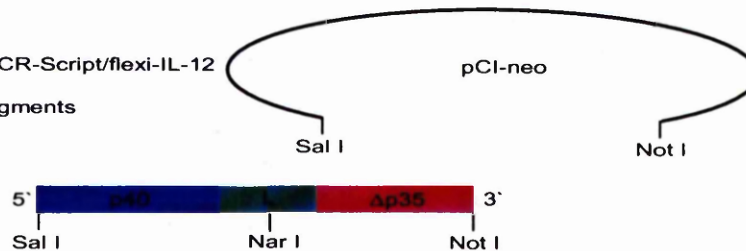
3) Nar I / Sal I digest
Purification of DNA
fragments



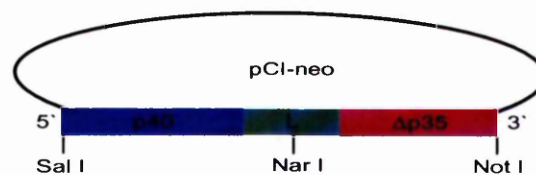
4) Ligation of DNA fragments



5) Sal I / Not I digest of pCR-Script/flexi-IL-12
and pCI-neo
Purification of DNA fragments



6) Ligation of DNA fragments



3.2.2 PCR of 5'/p40-linker/3' and 5'/linker-Δp35/3' fragments

Initially both *Taq* DNA polymerase and *Pfu* polymerase methods were attempted. However although *Taq* DNA polymerase produced successful amplification, the requirement of a polishing step to allow ligation of the *Taq* polymerase fragments into pCR-Script (Stratagene) greatly lowered the efficiency of ligation. Also, the potential for misincorporation of nucleotides of *Taq* enzyme led to the decision to pursue the *Pfu* polymerase reactions in the PCR of these fragments.

Template DNA encoding feline p40 and p35 sequence consisted of a pCI-neo plasmid containing both feline subunits under separate CMV promoters (RP31) which was kindly provided by Samantha Taylor (Retrovirus Research Laboratories, University of Glasgow). RP31 was cloned by the excision of a DNA fragment encoding a CMV/p40/poly A from an intermediate cloning vector using a *DraIII/AflIII* digest which was then filled in. A pCI-neo/CMV/p35/polyA construct was digested using *RsrII/DraIII* and ligation produced the final RP31 construct. The RP31 DNA template was diluted to a concentration of 100 ng/μl in dH₂O and 100 ng was used in the reaction.

Master mix reactions were prepared in 0.2 ml PCR reaction tubes on ice according to the instructions of the manufacturer. PCR reactions for both 5'/p40-linker/3' and 5'/linker-Δp35/3' were performed in a Perkin Elmer 9600 thermal cycler under the following conditions: 94°C for 45 seconds, 30 cycles of 94°C for 45 seconds, 40°C (annealing temperature) for 45 seconds, and 72°C for 2 minutes. This was terminated with a single cycle of 72°C for 10 minutes and reactions were maintained at 4°C. PCR products were visualised on a 0.8% ethidium bromide agarose gel using 10 μl of reaction per well diluted to 20 μl with dH₂O and 5 μl of DNA loading buffer.

Under these conditions, a fragment of the correct size (692 bp) was produced for 5'/linker-Δp35/3'. However there was no amplification of 5'/p40-linker/3'. Therefore reaction conditions were varied by altering the p40 primer levels and the amount of DNA template as follows:

Table 3-1 Range of reaction conditions used for amplification of 5'/p40-linker/3'

Reaction	Primer (μ M)	DNA (ng)
1	100	100
2	75	100
3	25	100
4	100	25
5	75	25
6	25	25

A primer concentration of 25 μ M and a DNA level of 25 ng produced successful amplification of a DNA fragment of the appropriate size (1053 bp) on UV visualisation of a 0.8% agarose gel.

3.2.3 Cloning of PCR fragments into the intermediate pCR-Script vector

The pCR-Script Amp Cloning Kit (Stratagene) was used to clone PCR fragments according to the manufacturer's instructions. A purification step separated the PCR products from primers, enzymes, buffer and unincorporated nucleotides associated with the PCR reaction. Purification used a microspin cup containing silica-based matrix that binds DNA. The DNA was washed and eluted in low-ionic strength buffer and 8 μ l of the purified PCR product was used in the ligation into the vector.

The ligation was prepared in a 0.5ml microfuge tube by mixing reagents in the following order: 10 ng pPCR-Script vector, 1 x reaction buffer, 5×10^{-3} μ M rATP, 8 μ l (around 200 ng) PCR product, 5 U *Srf*I restriction enzyme and 4 U T4 DNA ligase made up to a final volume of 10 μ l with dH₂O. The use of *Srf*I enzyme in the ligation preserves a high concentration of digested vector, increasing the efficiency of the reaction. The ligation was incubated at room temperature for 1 hour, 65°C for 10 minutes and then stored on ice until the transformation reaction.

The ligation product was transformed into Epicurian Coli XL10-Gold Kan ultracompetent cells according to the manufacturer's instructions. Blue/white colour screening was performed as described in 2.2.9. Colonies were selected and

minipreparations of DNA were prepared. 5'/linker- Δ p35/3' (sense) clones were identified using a *Bam*H I restriction digest and 5'/p40-linker/3' (non-sense) clones were identified with an *A*fIII/*Sac*II double restriction digest. These digested reactions were visualised on a 0.8% agarose gel stained with ethidium bromide.

3.2.3.1 Sequencing of positive clones

Positive clones were subcultured overnight at 37°C by seeding 30 μ l cultured broth into 3 ml LB medium containing 100 μ g/ml ampicillin. High-quality DNA for sequencing was prepared using Qiaprep Miniprep Kit (Qiagen). The DNA was quantified on a 0.8% agarose gel with ethidium bromide and cycle sequencing reactions were prepared using 500 ng DNA per reaction. T7 and M13-Reverse primers situated at the 5' and 3' ends of the insertion site for pCR-Script were used to sequence vector inserts.

Primer	Sequence (5'-3')
T7	TAATACGACTCACTATAGGG
M13-Reverse	GGATAACAATTCACACAGG

The Li-Cor 4000 model was initially used for flexi-IL-12 sequencing, but G/C rich regions of the gene yielded inaccurate sequence data. The ABI Prism technique was therefore used in these problem areas. Consensus sequences were produced for each clone and compared with the source IL-12 sequence to detect any ambiguities. A pCR-Script/5'/linker- Δ p35/3' clone and a pCR-Script/5'/p40-linker/3' clone with no ambiguities were then used for ligation to produce pCR-Script/flexi-IL-12.

3.2.4 Ligation of 5'/p40-linker/3' and pCR-Script/5'/linker- Δ p35/3'

5'/p40-linker/3' fragment was excised from the pCR-Script vector using a bulk digest of *Nar* I/*Sal* I. In addition, a *Nar* I/*Sal* I bulk digest of pCR-Script/5'/linker- Δ p35/3' excised a 54 bp fragment and opened the plasmid at the 5'/linker area to allow ligation with 5'/p40-linker/3'. Fragments were purified and ligated as described in 2.2.6 and 2.2.7. The ligation reaction was transformed into Epicurian Coli XL10-Gold

Kan ultracompetent cells according to the instructions of the manufacturer. Colonies were selected and minipreparations of DNA were prepared as described in 2.2.2. Positive pCR-Script/flexi-IL-12 clones were identified using *Not* I/*Sal* I, *Bgl* II and *Pvu* I/*Nde* I digests.

3.2.5 Ligation of flexi-IL-12 Into pCI-neo

A glycerol stock of a pCR-Script/flexi-IL-12 positive clone was prepared, streaked out onto an LB agar plate using a sterile wire loop and incubated overnight at 37°C. A single colony was selected and DNA was prepared as detailed in 2.2.1. *Not* I/*Sal* I bulk digests of pCR-Script/flexi-IL-12 and pCI-neo allowed flexi-IL-12 and linearised pCI-neo fragments to be isolated and purified as described in 2.2.6. A ligation reaction was performed and transformed into Epicurian Coli XL10-Gold Kan ultracompetent cells according to the instructions of the manufacturer. Single colonies were selected and minipreparations of DNA were prepared. Positive clones were confirmed using *Bgl* II digest. DNA was prepared from a positive clone using Qiagen plasmid Mega Kit (Qiagen) following the instructions of the manufacturer and glycerol stocks of the final construct were prepared and stored at -70°C.

3.2.5.1 Sequencing of pCI-neo/flexi-IL-12

Sequencing of the final flexi-IL-12 construct was performed using the ABI Prism technique following the protocol described in 2.2.14.2. T7 and T3 primers incorporated in the pCI-neo plasmid were used to sequence the 5' and 3' ends of the construct and specific primers were designed within the flexi-IL-12 sequence (figure 3.5).

Primer	Sequence (5'-3')
T7	TAATACGACTCACTATAGGG
T3	CTTCCTTAGTGAGGGTTAAT

The data was analysed using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin). Authentic sequence of pCI-neo/flexi-IL-12 was further confirmed by analysing the chromatogram by eye.

Figure 3-5 Sequence of flexi-IL-12 and position of sequencing primers

Red text	- primer derived sequence	Black text	- feline p40
Blue text	- glycine-serine linker sequence	Grey text	- feline Δ p35
Green text	- forward primers	Purple text	- reverse primers

```

1  gcggttaacg tcgacacgcc acgatggatc ctcagcagct ggtcatcgcc
51  tggttttccc tggttttgct ggcacctccc ctcattggcca tatgggaact
101 ggagaaaaac gtttatgttg tagagttgga ctggcaccct gatgcccccg
151 gagaaatggt ggtcctcacc tgcaatactc ctgaagaaga tgacatcacc
201 tggacctctg accagagcag tgaagtccta ggctctggta aaactctgac
251 catccaagtc aaagaatttg cagatgctgg ccagtatacc tgtcataaag
301 gaggcgaggt tctgagccat tcgttcctcc tgatacaca aaaggaagat
351 ggaatttggt ccaactgatat cttaagggaa cagaagaat ccaaaaataa
401 gatctttcta aaatgtgagg caaagaatta ttctggacgt ttcacctgct
451 ggtggctgac ggcaatcagt accgatttga aattcactgt caaagcagc
501 agaggctcct ctgacccccca aggggtgact tgtggagcag cgacactctc
551 agcagagaag gtcagagtgg acaacaggga ttataagaa gtacacagtgg
601 agtgtcagga gggcagtgcc tgcccggctg ccgaggagag cctaccatt
651 gaagtcgtgg tggacgctat tcacaagctc aagtacgaa actacaccag
701 cagcttcttc atcagggaca tcatcaaacc ggaccacccc aagaacctgc
751 aactgaagcc attaaaaaat tctcggcatg tggaagtgag ctgggaatac
801 cctgacacct ggagcacccc acattcctac ttctccttaa catttggcgt
851 acaggtccag ggcaagaaca acagagaaaa gaaagacaga ctctccgtgg
901 acaagacctc agccaaggtc gtgtgccaca aggatgcaa gatccgcgtg
951 caagccagag accgctacta tagctcatcc tggagcaact gggcatccgt
1001 gtcctgcagt ggtggcgggtg gtcggggcgg tgggtggatcg ggcgcgggtg
1051 gcggtatctag gaacctcccc acaccacac caagcccagg aatgttccag
1101 tgctcaacc actcccaaac cctgctgcga gccatcagca acacgttca
1151 gaaggccaga caaactctag aattttactc ctgcacttcc gaagagattg
1201 atcatgaaga tatcacaaaa gataaaacca gcacagtgga ggctgctta
1251 ccaactggaat taaccatgaa tgagagtgc ctggcttcca gagagatctc
1301 tctgataact aatgggagtt gcctggcctc cagaaagacc tcttttatga
1351 cgacctgtg ccttagcagt atctatgagg acttgaagat gtaccagggtg
1401 gagtccaagg ctatgaatgc aaagctgtta atggatccta aaaggcagat
1451 ctttctggat caaaacatgc tgacagctat tgatgagctg ttacaggccc
1501 tgaatgtcaa cagtgtgact gtgccacaga actcctccct ggaagaacca
1551 gatttttata aaactaaaat caagctctgc atacttcttc atgctttcag
1601 aattcgtgca gtgaccatca atagaatgat gagctatctg aatgcttctt
1651 aagcggccgc gttaacgagc tc

```

3.2.6 *In vitro* expression of flexi-IL-12 protein

3.2.6.1 Transfection of flexi-IL-12 into 293T cell line

In order to demonstrate *in vitro* expression of the flexi-IL-12 plasmid, transfections were performed using 293T cells. Several other constructs were transfected alongside flexi-IL-12 as detailed in the table below. The cloning of RP31 is described in 3.2.2.

Table 3-2 Feline IL-12 plasmic constructs used for *in vitro* expression

Construct	Description	Plasmid	Promoter(s)	Source
flexi-IL-12	Feline p40 + Δ p35 linked by a glycine-serine rich coding region	pCI-neo	CMV + intron	Constructed as previously described
RP31	Feline p40 and p35	pCI-neo	p35: CMV + intron p40: CMV	S. Taylor*
pCI-neo/p40	Feline p40 subunit	pCI-neo	CMV + intron	L. Hanlon**
pCI-neo/p35	Feline p35 subunit	pCI-neo	CMV + intron	L. Hanlon**

* Retrovirus Research Laboratories, University of Glasgow.

** Molecular Oncology Laboratories, University of Glasgow.

10 μ g each of flexi-IL-12, RP31, pCI-neo/p40, pCI-neo and 5 μ g each of pCI-neo/p35 and pCI-neo/p40 DNA were transfected using 75 cm² flasks as described in 2.2.15.5. A negative control consisting of cells transfected with empty pCI-neo plasmid was prepared alongside. Cells were transfected using LipofectAMINE Reagent (Invitrogen Life Technologies, UK) using the protocol given by the manufacturer. They were then incubated for 72 hours at 37°C with 5% CO₂.

Supernatant and cell lysate were then harvested from each transfection. Supernatant was removed from the cells, centrifuged at 1000 rpm for 5 minutes to remove any cellular debris, aliquoted and stored at -20°C until further use. The cells were washed with 1 x PBS and incubated at 37°C in 4 ml trypsin-EDTA until they had detached from the flask bottom. A volume of 8 ml of fresh medium was added and the cells were centrifuged for 5 minutes at 1000 rpm. The pellet was resuspended in 2 ml 1 x PBS with protease inhibitors (1 mM AEBSF and 10 μ g/ml Leupeptin). Cells were

then frozen and thawed three times using a dry ice/ethanol mix in order to rupture the cell membranes and release the protein into the surrounding buffer. This cell lysate was centrifuged for 30 seconds at 14000 rpm to pellet cell debris and the lysate was removed and stored at -20°C until further use.

3.2.6.2 Expression of flexi-IL-12 protein using Western blot analysis

In vitro protein expression of flexi-IL-12 in cell lysate and supernatant of transfected 293T cells was investigated using Western blot analysis. The primary antibody used for this procedure was a rabbit anti-feline p40 peptide antibody (Intervet International) delivered at a dilution of 1:1500. This antibody was generated to a peptide derived from a 17 amino acid region of feline p40 sequence. The secondary antibody was anti-rabbit HRP conjugate (Sigma), which was used at a dilution of 1:3000.

A 12% SDS-PAGE gel was prepared under denaturing conditions as described in 2.2.10. A volume of 20 µl each of flexi-IL-12, pCI-neo, RP31 and pCI-neo/p40 supernatant and cell lysate were prepared as described. The positive control of equine flexi-IL-12 supernatant was used, which demonstrates protein of the same expected size as the feline flexi protein. Feline flexi IL-12 supernatant was concentrated 10 fold as protein bands were only just within the detectable limits of this system. This was performed using a centrifugal filter technique (Centriplus) to remove proteins greater than 100KDa and was carried out according to the instructions of the manufacturer.

3.2.7 *In vitro* bioactivity of flexi-IL-12 using IL-12 bioassay

3.2.7.1 Preparation of assay plate

This bioassay was originally developed by Sam Taylor (Retrovirus Research Laboratories, University of Glasgow), for assessing the bioactivity of equine flexi-IL-12 [McMonagle *et al.* 2001] but was also found to be sensitive to feline IL-12 protein. LN156 cells were recovered from equine lymph nodes and placed in liquid nitrogen as described in 2.2.15.4. Feline cells were not used in this assay because feline lymph

nodes were difficult to obtain and produced low yields of lymphocyte populations compared to equine lymph nodes.

On revival, the cells were thawed quickly in a water bath at 37°C, slowly resuspended in 10 ml culture medium and centrifuged at 1000 rpm with low brake for 5 minutes. The cells were slowly resuspended in 10 ml growth medium and counted using a haemocytometer. Cell density was adjusted to 1×10^6 /ml and supplemented with 1 µg/ml of PHA-L (Sigma, UK), and human IL-2 (T. Dunsford, Retrovirus Research Laboratories, Department of Veterinary Pathology, University of Glasgow). 100 µl of cell suspension was administered to each well of a 96 well plate and this was incubated for 24 hours at 37°C with 5% CO₂.

Ten fold dilutions of flexi-IL-12, RP31, pCI-neop35/p40 and pCI-neo 293T transfection supernatants were made in LN156 medium (RPMI 1640 with no glutamine, 4 mM L-Glutamine, 1 x penicillin/streptomycin, 10% horse serum, 57.2 µM 2-ME and 20 µg/ml gentamicin) from 10⁻² to 10⁻⁹. In addition, another two sets of dilutions of Flexi-IL-12, RP31 and pCI-neop35/p40 were prepared alongside. Added to one set was a feline IL-12 cross reactive monoclonal antibody (C8.6 IgG₁ mouse anti-human IL-12 p40/p70 (BD PharMingen)) added to each dilution at a level of 10 ng/ml. With the other set of dilutions was incubated a negative control antibody (mouse monoclonal IgG₁ anti-protein A antibody (Sigma)) also added at a concentration of 10 ng/ml. As a negative control, pCI-neo transfection was prepared alongside at the same dilutions. These dilutions were incubated for 2 hours at 37°C with 5% CO₂ and 100 µl of each dilution was added to wells in triplicate and mixed gently. LN156 cells were incubated for 72 hours at 37°C with 5% CO₂ and amounts of IFNγ produced by each sample was measured using an IFNγ sandwich ELISA.

3.2.7.2 IFNγ sandwich ELISA

Three antibodies were used in this procedure. The capture SE3 antibody was an affinity purified sheep polyclonal antibody, raised to recombinant equine IFNγ. Primary R1053E2 antibody was a rabbit polyclonal raised to bacterially produced

recombinant equine IFN γ . The secondary antibody was goat anti-rabbit HRP conjugate (Sigma).

A high binding 96 well plate (Corning Incorporated) was incubated for 24 hours at room temperature with SE3 capture antibody. The antibody was diluted 1:500 in coupling buffer and 100 μ l was added per well. Wells were blocked for 20 minutes with 200 μ l of 0.1% casein in TBS-T buffer, on a shaking platform at room temperature. Each well was washed twice with TBS-T and 100 μ l of samples, including zeroes and standards, were added and incubated for 2 hours, shaking at room temperature. Standards consisted of recombinant equine IFN γ diluted 1:2 from 15.6 ng/ml to 0.122 ng/ml in LN156 culture medium. The plate was washed four times as before and then incubated for 2 hours with 1:1000 dilution of primary antibody, shaking at room temperature. After washing four times, the secondary antibody was added at a dilution of 1:1000 for 1 hour, with gentle shaking at room temperature. The washing was repeated and 100 μ l TMB liquid substrate was added to each well and incubated for 30 minutes at room temperature. The plates were read using a spectrometer at 620 nm and the results were analysed using a Microsoft Excel worksheet.

3.3 RESULTS

3.3.1 Amplification of 5`/Δp35-linker/3`

The PCR reaction using *Pfu* reagents for Δp35 amplification was run on a 0.8% agarose gel and displayed a band of the expected size (692 bp) (figure 3.6). The fragment was cloned into pCR-Script and sequenced. No mutations were found in the plasmid, so this clone was used in the subsequent ligation with 5`/p40-linker/3`/PCR-Script.

3.3.2 Amplification of 5`/linker-p40/3`

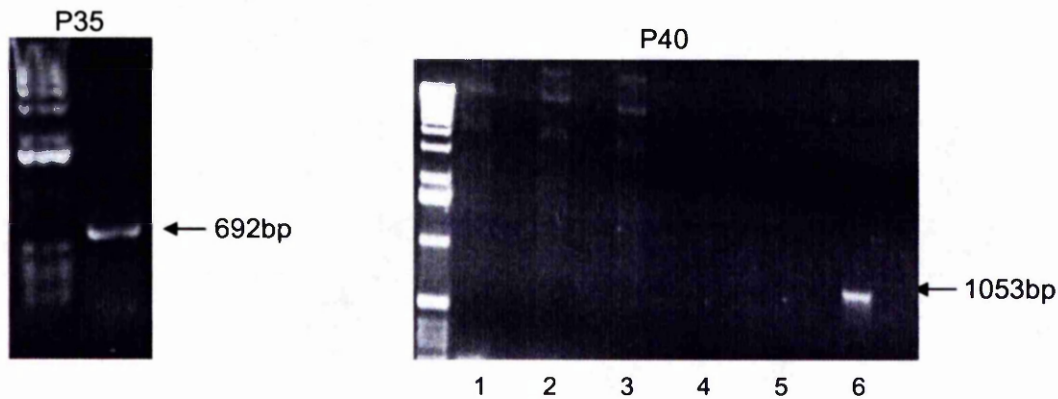
Of the 6 reactions with variable primer and DNA template concentrations, it was found that a primer concentration of 25 μM and a DNA concentration of 25 ng/μl produced successful amplification of a DNA fragment of the appropriate size (1053 bp) (figure 3.6). The fragment was successfully cloned into pCR-Script and sequenced. Of two clones sequenced, one had a substitution mutation and was abandoned. The other was free of ambiguities and was subsequently used in the ligation to produce the full-length flexi-IL-12 sequence.

3.3.3 Ligation of flexi-IL-12 and cloning of flexi-IL-12 into pCI-neo

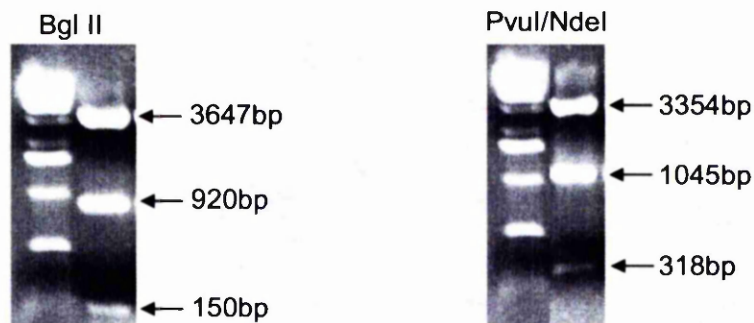
Restriction digests *Bgl* II and *Pvu* I/*Nde* I were used to identify positive flexi-IL-12/PCR-Script clones as shown in figure 3.6. Positive flexi-IL-12/pCI-neo clones were detected using *Bgl* II restriction digests (figure 3.6) and a single positive clone was sequenced using internal primers (figure 3.5). Plasmid DNA was prepared from this clone and used in subsequent transfections to show *in vitro* expression.

Figure 3-6 Gels of feline flexi-IL-12 cloning strategy

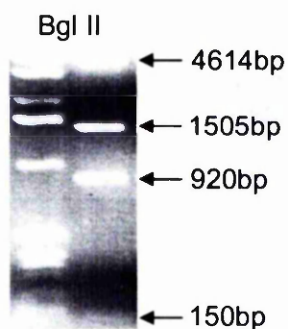
Amplification of PCR fragments



Restriction digests of 5'/p40-linker-p35/3'/PCR-Script



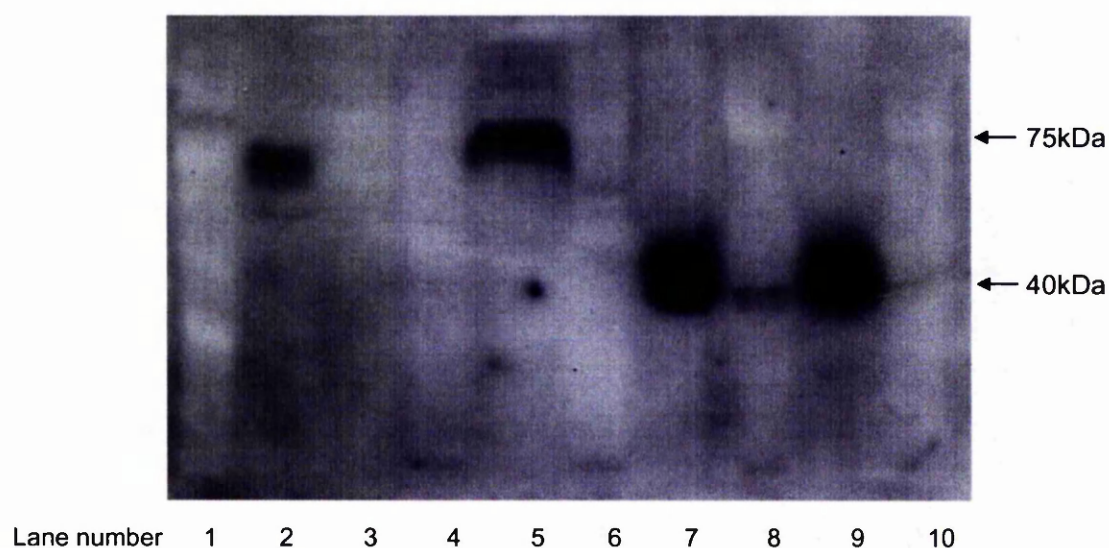
Restriction digests of 5'/p40-linker-p35/3'/pCI-neo



3.3.4 *In vitro* expression of flexi-IL-12

Supernatants and cell lysates of flexi-IL-12, RP31 and pCI-neo/p40 transfections were analysed on a 12% SDS-PAGE gel (figure 3.7).

Figure 3-7 SDS-PAGE gel of feline flexi-IL-12 protein



This is an autoradiograph of a 12% SDS-PAGE gel depicting feline IL-12 p40 subunit and feline flexi-IL-12 protein. Each lane contains 20 μ l of the following:

lane 1: kaleidoscope protein marker, **lane 2:** equine flexi-IL-12 supernatant, **lane 3:** pCI-neo supernatant, **lane 4:** pCI-neo cell lysate, **lane 5:** feline flexi-IL-12 supernatant 10 x concentrate, **lane 6:** feline flexi-IL-12 cell lysate, **lane 7:** RP31 supernatant, **lane 8:** RP31 cell lysate, **lane 9:** feline p40 supernatant, **lane 10:** feline p40 cell lysate.

Flexi-IL-12 supernatant contained a protein of approximately 75 kDa corresponding to the expected size of p40 (40 kDa) linked to p35 (35 kDa) via a synthetic linker sequence. This protein product was a similar size to that seen in the equine flexi-IL-12 supernatant positive control [McMonagle *et al.* 2001]. There was no evidence of a protein product of 40 kDa, suggesting that the fusion protein was stable and there was no breakdown into the separate subunits.

The supernatant of RP31 plasmid transfection contained a double protein band at approximately 40 kDa with no evidence of full length IL-12 protein at 75 kDa. As explained previously, the primary antibody would not have detected any p35 protein expressed by this plasmid. A similar p40 protein band 40 kDa in size was evident in the feline p40 supernatant.

There were no bands evident in the cell lysate of feline flexi-IL-12. Only faint 40 kDa bands were evident in RP31 and feline p40 plasmid cell lysates, implying that IL-12 related protein is secreted and not retained within the cell from which it is expressed.

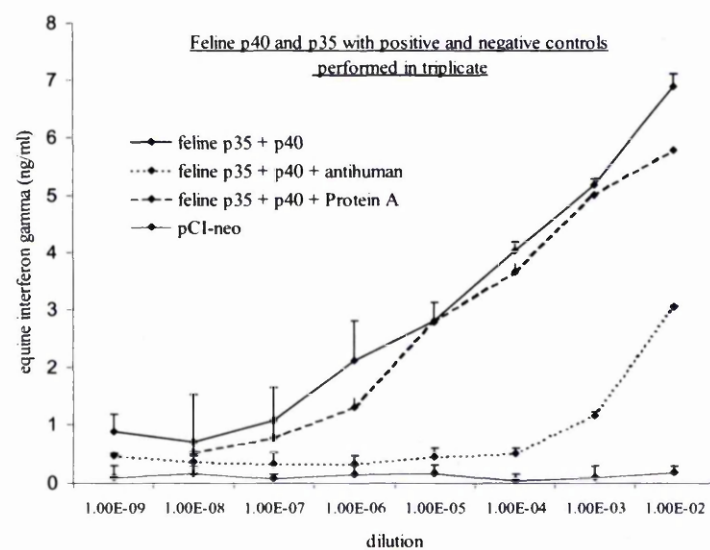
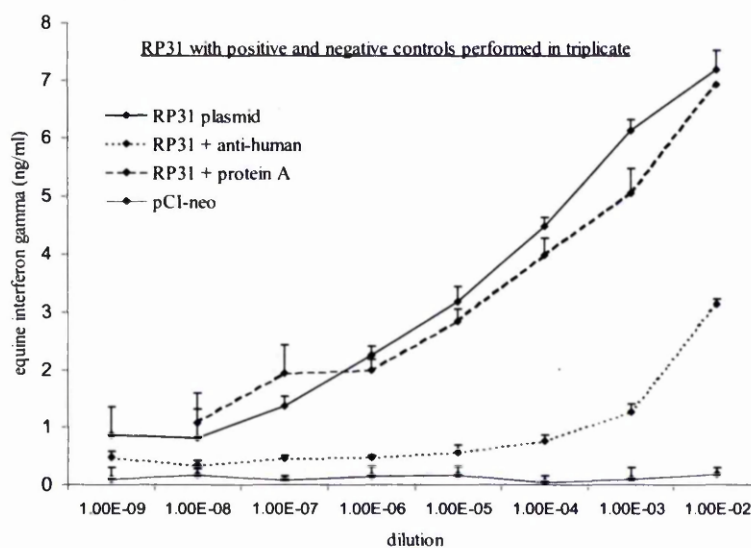
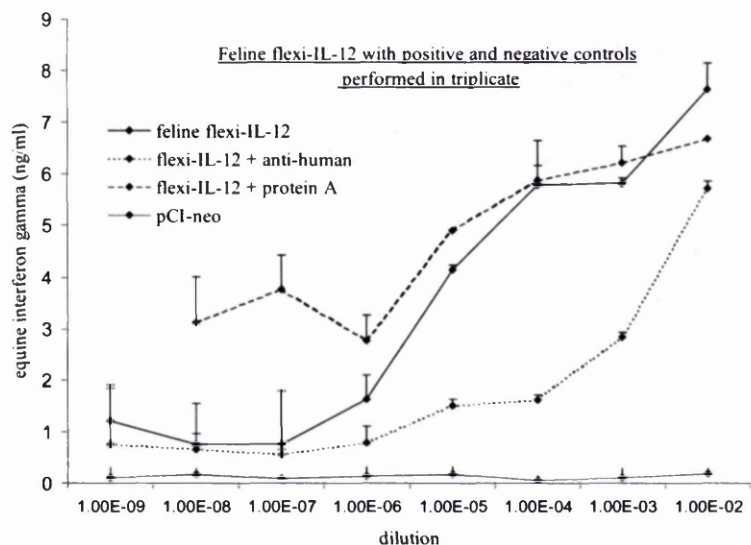
3.3.5 *In vitro* bioactivity of flexi-IL-12/pCI-neo using IL-12 bioassay

The bioactivity of transfection supernatants was analysed using a bioassay that measures the amount of equine IFN γ produced in response to IL-12 by LN156 cells isolated from equine lymph nodes. The assay was performed using ten fold dilutions of protein and IFN γ produced was quantified by capture ELISA. The OD of samples and human IFN γ standards were measured using a spectrophotometer.

Analysis of flexi-IL-12, RP31, pCI-neo/p40/p35 and pCI-neo transfections were carried out using this assay. In addition, to confirm that IFN γ stimulation was specific to IL-12 expression, dilutions of supernatant produced from 293T cells transfected with each construct were incubated with an anti-human IL-12 antibody. This study demonstrated that anti-human IL-12 antibody induced suppression of IFN γ production suggesting cross-reactivity with feline IL-12. Anti-protein A, a negative antibody non-specific to IL-12 was used as a negative control.

A standard curve was constructed of human IFN γ (ng/ml) against OD using the sample standards performed on the capture ELISA plate. Graphs were then constructed of IFN γ against dilution of protein for flexi-IL-12, RP31, pCI-neo/p40/p35 transfections and their associated control data (figure 3.8).

Figure 3-8 Graphs of IL-12 bioassay



The quantity of IFN γ produced by LN156 cells decreased as the dilution of feline flexi-IL-12 protein increased, producing an S-shaped dose response curve. In contrast, pCI-neo transfection supernatant stimulated undetectable levels of IFN γ production. When flexi-IL-12 protein was preincubated with anti-human IL-12, this significantly reduced the level of IFN γ induction, confirming that the antibody recognised feline IL-12 and that bioactivity was specific to IL-12 protein present in the transfection supernatant. The negative control anti-protein A antibody had no significant effect on the bioactivity of flexi-IL-12. This indicates that suppression of IFN γ induction by the anti-human antibody was due to binding and inactivation of IL-12 rather than non-specific antibody neutralisation. A similar level of bioactivity and suppression by anti-human antibody was also demonstrated in both RP31 and p40/p35 transfection supernatants as shown in the appropriate graphs.

It has been shown that IL-12 p35 is constitutively expressed in many cells in the absence of p40 [D'Andrea *et al.* 1992; Schoenhaut *et al.* 1992]. Levels of endogenously generated p35 could not be quantified in these experiments, as there was no p35 antibody available. It is therefore a theoretical possibility that the protein expressed from transfected p40 cDNA could complex with endogenous p35 to produce functional IL-12 affecting IFN γ levels stimulated by the bioassay. Although not conducted here, this possibility was investigated in the equivalent equine IL-12 bioassay where equine p40 transfection supernatants were associated with only background IFN γ levels. This suggests that endogenous p35 does not contribute to IL-12 detected by this bioassay system.

3.4 DISCUSSION

This chapter presents the cloning, *in vitro* expression and confirmation of bioactivity of feline flexi-IL-12 expressed in mammalian expression vector pCI-neo. PCR fragments encoding feline p40 and p35 subunits of IL-12 were linked using a synthetic linker sequence built into specifically designed primers. Restriction sites included in primers allowed ligation to form the flexi-IL-12 fragment, which was subsequently cloned into pCI-neo.

Western blot analysis using a rabbit anti-feline p40 peptide antibody, demonstrated *in vitro* expression of a 75 kDa sized protein molecule. In order to investigate *in vitro* bioactivity of this protein, a bioassay previously used to study equine IL-12 constructs was used [McMonagle *et al.* 2001]. Flexi-IL-12 protein expressed in transfected cells was shown to be bioactive and exhibit similar levels of IFN γ induction in equine lymph node derived cells as naturally formed IL-12 protein.

3.4.1 Cloning of flexi-IL-12/pCI-neo

The use of a polypeptide synthetic linker to unite p40 and p35 subunits of IL-12 originated from Lieschke *et al.* [1997] and Anderson *et al.* [1997]. In these studies, IL-12 fusion protein was expressed using a linker previously used to connect subunits of antibody molecules [Huston *et al.* 1988]. Lieschke *et al.* found that murine fusion protein was bioactive *in vitro*, having a specific activity equivalent to that of native and recombinant IL-12 [Lieschke *et al.* 1997]. Anderson *et al.* confirmed that the human fusion protein retained all the biological characteristics of recombinant IL-12 [Anderson *et al.* 1997].

A comparison of the bioactivity of different murine IL-12 constructs was also made by assaying the bioactive IL-12 produced by CMS-5 tumour cells infected with protein from different IL-12 construct transfections [Lieschke *et al.* 1997]. Various IL-12 constructs were cloned into retroviral vectors including separate p35 and p40 plasmids, IRES constructs and flexi-IL-12 constructs. Of these, the flexi-IL-12 plasmid with 5'p40 showed the highest level of IL-12 in the bioassay and the highest

relative specific activity. The feline flexi-IL-12 plasmid was therefore designed on the basis of these results.

One of the major difficulties encountered in this cloning strategy involved the application of PCR to amplify 5'/p40-linker/3' and 5'/linker-p35/3' fragments. Initially PCR reactions were performed using *Taq* DNA polymerase and *Pfu* polymerase alongside one another.

Taq DNA polymerase reactions yielded immediate amplification of the appropriately sized DNA fragments. However, this method was ultimately rejected for several reasons. Firstly, as *Taq* tends to add non-template nucleotides to the 3' end of DNA fragments, a polishing step using *Pfu* polymerase was required to clone fragments into PCR-Script. This polishing step was found to greatly decrease the efficiency of ligation, with bacterial transformations yielding predominantly plasmid vector without insert. In addition, *Taq* DNA polymerase is a low fidelity enzyme with a higher error rate than *Pfu*, estimated to be 2×10^{-5} [Lundberg *et al.* 1991]. This error rate may also increase if cycling conditions are not optimal for the enzyme. This has led to the recommendation that between three and six amplified fragments are required to establish a consensus sequence [Ennis *et al.* 1990]. This, along with low *Taq* ligation efficiency resulted in the subsequent pursuit of fragments amplified using *Pfu* polymerase. This enzyme produces 11-12-fold greater replication fidelity than *Taq*, with an error rate of 1.6×10^{-6} [Lundberg *et al.* 1991] and produces blunt ended fragments that do not require a polishing step prior to ligation.

Pfu DNA polymerase produced successful amplification of 5'/linker-p35/3' clone containing no sequence ambiguities. However manipulation of both primer and DNA template concentrations were required in order to amplify 5'/p40-linker/3' fragment. A primer concentration of 25 μ M and a DNA concentration of 25 ng/ μ l resulted in production of a 5'/p40-linker/3' clone which was used to generate full-length flexi-IL-12.

3.4.2 *In vitro* expression of flexi-IL-12 protein

pCI-neo plasmid encoding flexi-IL-12 was transfected into 293T cells and the protein detected under denaturing conditions by Western blot using a rabbit anti-feline peptide antibody raised to p40. A 75 kDa band was detected in the cell supernatant but not in the cell lysate. This suggests that once synthesised, IL-12 protein is rapidly secreted from the cell into its surroundings. Any protein present within the cells is in insufficient quantities to be detected by this method. No p40 subunit protein was detected in flexi-IL-12 supernatant. This suggests that flexi-IL-12 is stable under denaturing conditions with no breakdown into separate subunits.

The 75kDa band in the neat flexi-IL-12 supernatant was almost undetectable and required concentration by ten-fold in order to detect the protein adequately on immunoblot. In comparison, feline p40 expressed by both RP31 and feline p40 supernatants was detectable without concentration. The reason for this may be that the flexi-IL-12 is glycosylated differentially from the native molecule which may affect binding of the primary antibody. Alternatively flexi-IL-12 may be less stable *in vitro* or be expressed in lower quantities than the p40 subunit.

As mentioned, the supernatants of both RP31 and feline p40 contained detectable levels of p40. Faint bands were also detected in the cell lysates of each transfection, perhaps due to the high degree of cellular expression of this protein. There was no evidence of disulphide linked IL-12 in the RP31 supernatant. This is to be expected, as denaturing conditions would cleave the protein into separate subunits. However, unlike feline flexi-IL-12 supernatant, protein in the RP31 supernatant was not concentrated further, so there is the possibility that the levels of protein in the neat supernatant were too low to be detected. This gel was repeated under non-denaturing conditions but no IL-12 was detected, possibly due to larger size and secondary structure of IL-12 as mentioned above.

3.4.3 Bioactivity of flexi-IL-12 protein

A bioassay performed previously to analyse bioactivity of equine IL-12 [McMonagle *et al.* 2001] was found to be sensitive to feline protein. Flexi-IL-12, RP31 and p40/p35 supernatants showed significant titration of LN156 IFN γ induction between 10^{-2} and 10^{-7} dilutions. Suppression of IFN γ production was elicited when IL-12 supernatants were incubated with anti-human IL-18 antibody which suggests that this antibody binds to and neutralises feline IL-18. In this assay, flexi-IL-12 supernatant was found to stimulate equivalent levels of IFN γ from LN156 cells as RP31 supernatant, where subunits synthesised separately became linked by a disulphide bond. However, a direct comparison between the bioactivity of flexi-IL-12 and disulphide linked IL-12 cannot be made for several reasons. Firstly, disulphide-linked IL-12 from RP31 could not be detected on Western blot which prevented comparison of relative amounts of flexi-IL-12 and RP31 IL-12 in transfections. In addition, free p40 present in RP31 supernatant may have induced an inhibitory effect on the IL-12 protein present, preventing a fair comparison of bioactivity of each molecule. The detection of bioactivity of flexi-IL-12 does however confirm that the synthetic polypeptide linker allows correct alignment of subunits and its structure does not abrogate the biological function of the molecule.

3.4.4 Advantages of flexi-IL-12 fusion protein

There are several reasons why flexi-IL-12 may be favoured over other IL-12 constructs. Firstly the use of a linker sequence reduces the size of the construct. This is particularly advantageous if used in viral vectors where construct size may be restricted.

In addition, use of the flexi-IL-12 construct ensures the equimolar production of both p35 and p40 within the same cell. The exposure of cells to p35 and p40 DNA in separate plasmids or IRES constructs does not guarantee equimolar subunit concentrations. In both cases, differential expression of IL-12 subunits has the potential to lead to decreased IL-12 protein production.

By linking p35 and p40 subunits in this way, the production of p40 homodimer is prevented. As previously mentioned, certain cells such as stimulated PBMCs, neutrophils and EBV transformed cell lines have been shown to express between 10-50 times the amount of free p40 as p70 heterodimer [D'Andrea *et al.* 1992]. This p40 has been shown to form disulphide linked homodimers which can act as specific antagonists to heterodimeric IL-12 by binding to the IL-12 receptor [Ling *et al.* 1995]. In addition, culture supernatants containing murine p40 were found to inhibit the biological activity of IL-12 demonstrated in *in vitro* assays [Mattner *et al.* 1993]. The use of constructs that allow synthesis of separate IL-12 subunits could potentially elicit inhibition of IL-12 function by overproduction of p40, whereas the structure flexi-IL-12 prevents this from occurring. To date however there is no *in vitro* evidence to demonstrate this.

3.4.5 Applications of flexi-IL-12

Since the development of flexi-IL-12 fusion protein [Anderson *et al.* 1997; Lieschke *et al.* 1997], this construct has been cloned in other species and used effectively *in vitro* and *in vivo*.

In the mouse, flexi-IL-12 has been used to prevent the development of metastases in a poorly immunogenic murine neuroblastoma model. NXS2 neuroblastoma cells were genetically engineered to produce flexi-IL-12 and were injected into mice. On boosting with a low dose of IL-2 fusion protein and tumor-specific antibody followed by challenge with a lethal dose of NXS2 cells, five out of six mice were protected from metastasis. Injection with flexi-IL-12-expressing cells was associated with a partially protective CD8⁺ T cell response, which was further enhanced by the booster described above [Lode *et al.* 1998; Lode *et al.* 1999]. Additional studies using the same flexi-IL-12-expressing NXS2 cells demonstrated complete protection from liver and bone marrow metastases without additional boost of the cellular immune response [Balicki *et al.* 2000].

Porcine flexi-IL-12 has also been cloned and oral inoculation *in vivo* was shown to increase antigen-specific IgA and IgG in jejunal mucus. This construct was expressed

as recombinant protein and found to be bioactive by lymphoblast proliferation and IFN γ production. This bioactivity was not inhibited by the coadministration of p40 subunit [Foss *et al.* 1999]. Porcine flexi-IL-12 was also found to act synergistically with human IL-12 in the induction of IFN γ from T and NK cells *in vitro* [Domeika *et al.* 2002].

Transfections of equine flexi-IL-12 cloned into pCI-neo, produced dose dependent IFN γ stimulation in cells recovered from equine lymph nodes. IFN γ production was suppressed using anti-p40 monoclonal antibody showing that the response was specific to flexi-IL-12 fusion protein [McMonagle *et al.* 2001].

There are several studies describing the use of flexi-IL-12 fused with proteins to target cytokine delivery to tumour cells. Murine flexi-IL-12 was fused to a tumour-specific antibody anti-Her2/*neu* to allow selective targeting to this tumour antigen. This preparation was shown to elicit antitumour activity *in vivo* [Peng *et al.* 1999]. A similar method was used to target a marker of angiogenesis B-Fibronectin which accumulates around the vasculature of proliferating tumours. An antibody to this marker was fused to murine flexi-IL-12 and displayed antitumour activity and infiltration of the tumour with immune cells [Halin *et al.* 2002].

Finally other IL-12 fusion constructs have been developed to ensure delivery of equimolar concentrations of each subunit to target cells. For example, a linker sequence encoding a cleavage site for furin, a Golgi expressed endoprotease, was included between IL-12 subunits in a single expression cassette. This construct was shown to be secreted from cells and to be bioactive by the stimulation of human T cell proliferation [Gäken *et al.* 2000].

A construct has also been designed which ensures equimolar levels of p35 and p40 protein by expression of bovine IL-12 as a self-processing 2A polypeptide [Chaplin *et al.* 1999]. 2A polypeptide is a 19 amino acid sequence derived from foot-and-mouth disease virus, which produces cleavage of the two subunits. This construct was subsequently shown to act as an adjuvant to a DNA vaccine for *Mycobacterium tuberculosis* [Palendira *et al.* 2002].

3.5 CONCLUSION

Recent work has shown the potential use of IL-12 DNA constructs in the treatment of cancer, immunotherapy and as adjuvants to vaccines. The requirement for the expression of two protein subunits from separate genes and the potential inhibition of IL-12 heterodimer by p40 homodimer, has recently led to the design of various new IL-12 plasmid constructs. Flexi-IL-12 has shown advantages over other constructs in that the subunits are fused, ensuring equimolar subunit expression and prevention of p40 homodimer inhibition. This chapter has described the cloning of feline flexi-IL-12 and *in vitro* expression and specific bioactivity of feline flexi-IL-12 protein. Overall this data supports the future use of this IL-12 fusion plasmid *in vivo*.

CHAPTER 4: INTERLEUKIN 18

4 INTERLEUKIN 18

4.1 INTRODUCTION

4.1.1 Isolation of interleukin-18

This cytokine was first discovered in 1989 when induction of IFN γ from mouse spleen cells was demonstrated by endotoxin-induced serum on costimulation with IL-2 and mitogens [Nakamura *et al.* 1989]. Neutralising antibodies to IL-1, IL-4, IL-5, IL-6 and TNF failed to suppress the activity of post-endotoxin mouse serum, which suggested that this effect was produced by a distinct factor [Nakamura *et al.* 1993]. This molecule, named IFN γ -inducing-factor (IGIF), was purified to homogeneity from mouse livers treated with *Propionibacterium acnes* and found to have a molecular mass of 18-19 kDa. IGIF was shown to augment T cell proliferation and NK cell activity in murine spleen cells in a similar way to IL-12 [Okamura *et al.* 1995b].

4.1.2 Molecular cloning of IGIF

The nucleotide sequence of murine IGIF was elucidated and the gene was shown to encode a precursor protein 192 aa in size and a mature protein of 157 aa [Okamura *et al.* 1995a]. Anti-IGIF antibodies were shown to prevent liver damage induced by LPS in *P. acnes* pre-conditioned mice [Okamura *et al.* 1995b].

The same group then cloned human IGIF cDNA from normal human liver cDNA libraries using murine IGIF cDNA as a probe. This encoded a 193 aa precursor peptide with 65% identity with murine IGIF at the amino acid level. The properties demonstrated by this peptide led to the proposal that IGIF be designated “interleukin-18” [Ushio *et al.* 1996]. The human interleukin-18 gene was shown to map to chromosome 11q22.2-q22.3, closely linked to the DRD2 gene locus [Nolan *et al.* 1998].

Full length canine IL-18 was shown to have 84% and 74% sequence homology to the human and mouse sequence respectively at nucleotide level [Argyle *et al.* 1999]. The bioactivity of a similar clone was confirmed by the induction of canine IFN γ from canine lymphocytes [Okano *et al.* 1999]. Feline IL-18 was cloned and found to be 192 aa in length with 77%, 85% and 63% identity at the amino acid level to human, canine and murine sequences respectively [Hanlon, 1999; Ishizaka *et al.* 2001].

4.1.3 Structure and processing of interleukin-18

Interleukin-18 is synthesised as a non-glycosylated biologically inactive precursor peptide called pro-IL-18, which is 24 kDa in size and lacks a conventional signal sequence [Okamura *et al.* 1995]. This molecule possesses similar structural homology to IL-1 β both of which are members of the IL-1 family [Bazan *et al.* 1996].

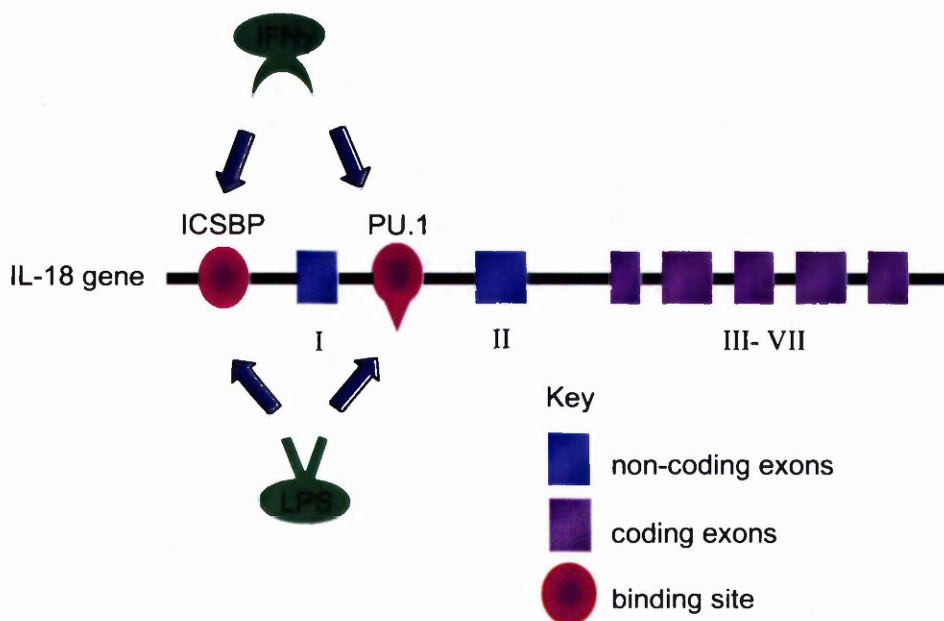
Pro-IL-18 is cleaved at Asp³⁵ in mice to produce the mature protein, which suggested that an aspartic acid-specific protease was involved [Okamura *et al.* 1995; Ushio *et al.* 1996]. It was found that interleukin-1 β converting enzyme (ICE or caspase-1) brought about cleavage of pro-IL-18 to mature IL-18 [Gu *et al.* 1997]. ICE is an intracellular cysteine protease, which is expressed by B cells, neutrophils and macrophages [Cerretti *et al.* 1992; Singer *et al.* 1995]. It is produced as a 45 kDa proenzyme which is cleaved to active ICE consisting of equal amounts of p10 and p20 subunits [Singer *et al.* 1995]. The proenzyme is cleaved into its mature form by either autoproteolytic conversion [Ramage *et al.* 1995], or by caspase-11 [Wang *et al.* 1998].

Caspase-3, also known as CPP32 is also able to cleave pro-IL-18 at Asp⁷¹ and Asp⁷⁶ producing biologically inactive peptide molecules [Akita *et al.* 1997] and a recent report has suggested that proteinase 3 may be an alternative enzyme for processing of IL-18 into its bioactive form [Sugawara *et al.* 2001].

4.1.4 Regulation of the IL-18 gene

The structure of the murine IL-18 gene has been elucidated and found to consist of 7 exons distributed over 26 kb (figure 4.1). Exons 1 and 2, situated at the 5' end are noncoding and distinct regions upstream from each noncoding exon show high promoter activity [Tone *et al.* 1997]. Each promoter is TATA-less and not G+C rich. The promoter upstream of exon 1 is inactive under normal conditions, being upregulated by activated macrophage and T cell lines, whereas the promoter upstream of exon 2 acts constitutively [Tone *et al.* 1997]. These promoters are used by many cell types, which may explain why IL-18 is expressed in both immune and non-immune cells [Nakanishi *et al.* 2001a]. The regulatory components of each promoter were examined and the promoter upstream of exon 1 required a binding site for the IFN consensus sequence binding protein (ICSBP), which is a member of the family of interferon regulatory factors, the binding of which was increased by LPS [Kim *et al.* 1999]. The expression of ICSBP has been shown to be upregulated by IFN γ [Wu *et al.* 1999]. A binding site for PU.1, a transcription factor that can also be upregulated by IFN γ as well as LPS [Shackelford *et al.* 1995], was found to be important for the activity of the promoter upstream of exon 2 [Kim *et al.* 1999].

Figure 4-1 Regulation of IL-18 gene



Once IL-18 protein is synthesised in cells it is stored as the inactive precursor pro-IL-18 [Gu *et al.* 1997]. This is in contrast to many other cytokines, which tend to have only a short half-life in the cell due to the processing of mRNA by RNA-destabilising elements [Cosman, 1987]. It has been suggested that the lack of RNA-destabilising elements allows storage of pro-IL-18 within these cells and that the active molecule is secreted via processing of this protein store [Nakanishi *et al.* 2001a].

4.1.5 Production of IL-18

IL-18 is produced by cells of the immune system and also non-immune cells. Certain cells require stimulation in order to produce expression of IL-18 protein. These include macrophages such as Kupffer cells [Okamura *et al.* 1995b], macrophages [Bohn *et al.* 1998], astrocytes and microglia [Conti *et al.* 1999] and keratinocytes in response to contact allergens [Stoll *et al.* 1997].

Other cells are able to secrete IL-18 in their normal functioning environment without stimulation. Enriched Langerhans cells and bone marrow-derived dendritic cell release functional IL-18 protein [Stoll *et al.* 1998], and human pro-IL-18 has been expressed constitutively in PBMCs from fresh whole blood [Puren *et al.* 1999].

4.1.6 IL-18 receptor

4.1.6.1 Structure

The receptor of IL-18 consists of a ligand-binding subunit termed IL-18R α and a signal transduction unit called IL-18R β . Human IL-18R α was identified using the Hodgkin's disease cell line L428 which was shown by binding assays to express high levels of IL-18 receptor [Torigoe *et al.* 1997]. The nucleotide sequence of the receptor was identical to human IL-1 receptor-related protein (IL-1Rrp) cDNA, a member of the IL-1 receptor (IL-1R) family which, when isolated, was found not to bind any of the known IL-1 ligands. The gene encoding human IL-18R α was found to be on chromosome 2q12-13, in a different location to IL-18 [Parnet *et al.* 1996; Nolan *et al.*

1998]. When a comparison of human and murine IL-18R α was made the overall amino acid identity was 65% [Parnet *et al.* 1996].

IL-18R β subunit is also a member of the IL-1R family, previously called IL-1R-accessory protein- like protein (AcPL) due to its similarity to IL-1R accessory protein. In a study, human IL-1Rrp and AcPL together were required to induce the IL-18 signalling pathway and AcPL alone was unable to bind IL-18, suggesting that AcPL was required for signal transduction whereas IL1Rrp was necessary for binding of the cytokine [Born *et al.* 1998].

4.1.6.2 IL-18 binding protein

Recently, a protein called IL-18 binding protein (IL-18BP) was isolated from human urine and was found to bind IL-18 and prevent IFN γ production in murine splenocytes [Novick *et al.* 1999]. This protein exists in 6 naturally occurring isoforms, four human and two murine forms, brought about by splicing of mRNA [Kim *et al.* 2000]. This protein functions as an inhibitor of the Th1 immune response and appears to show no species specificity, as human IL-18BP is able to bind murine IL-18 [Novick *et al.* 1999]. Work has shown that IL-18BP may be induced by IFN γ , which in turn limits the biological function of IL-18 [Paulukat *et al.* 2001].

4.1.6.3 Expression of IL-18 receptor

IL-18R α mRNA is expressed in lung, liver, heart, gut, prostate, placenta and thymus but not in brain, skeletal muscle, kidney and pancreas [Parnet *et al.* 1996]. The receptor has also been found in monocytic, erythroid, megakaryocytic, and myeloid cell lines [Nakamura *et al.* 2000].

IL-18R α has been shown to play a role in the synergism of IFN γ induction by IL-12 and IL-18. Treatment of T and B cells with IL-12 increased their responsiveness to IL-18. These cells were shown to demonstrate increased IL-18R α mRNA expression, particularly in T cells [Yoshimoto *et al.* 1998]. This increased responsiveness to IL-18

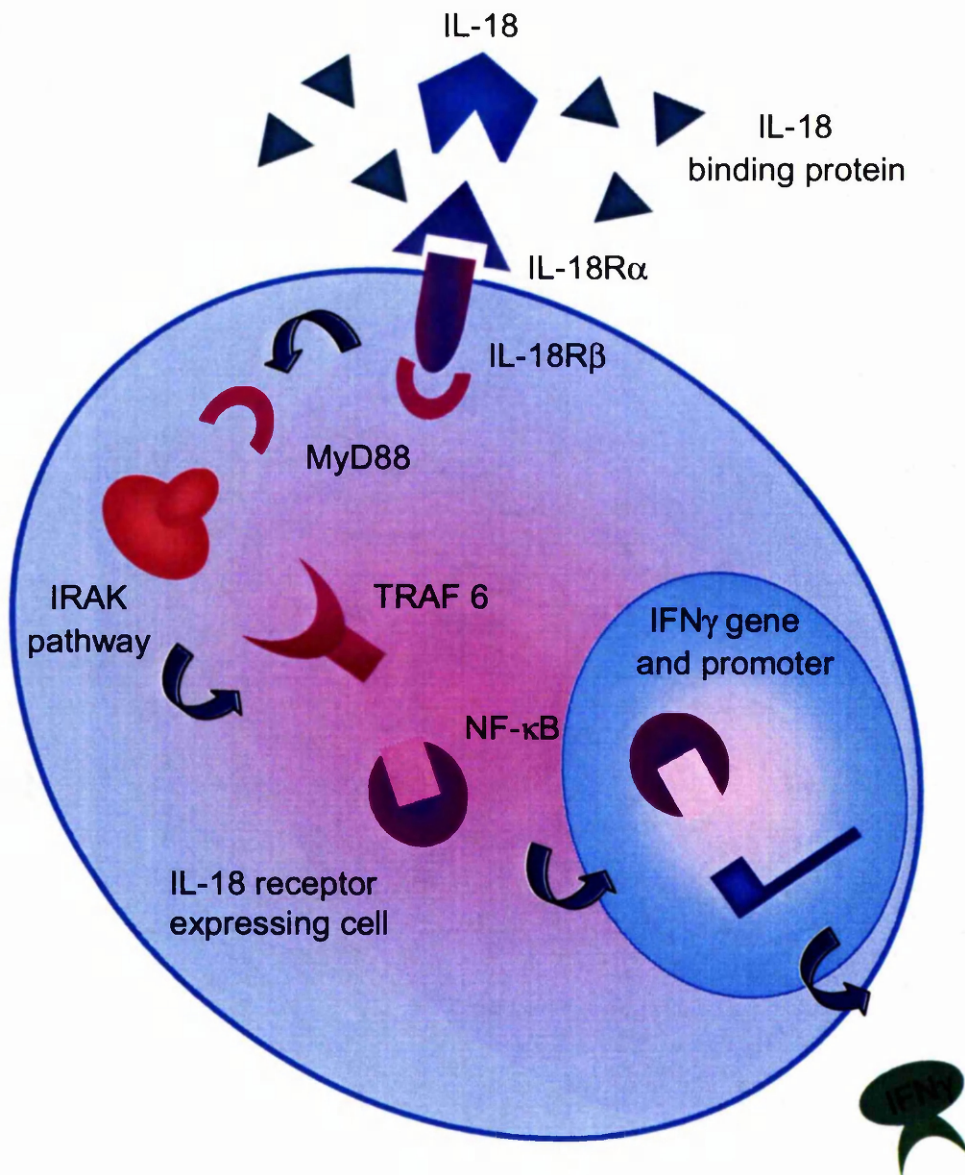
also increased the responsiveness to IL-12 by producing upregulation of IL-12R β 2 [Chang *et al.* 2000].

Different subsets of T cells demonstrate different levels of IL-18 receptor. In one study CD4⁺ T cells were shown to express marginal IL-18 receptor expression whereas CD8⁺ T cells expressed higher levels, producing more IFN γ in response to IL-18 than CD4⁺ cells [Tomura *et al.* 1998a]. One study showed that T and B cells express both high and low affinity IL-18 receptors, which produce IFN γ in response to IL-18 [Yoshimoto *et al.* 1998]. A recent study suggested that IL-1R β is required for the production of high affinity receptors for IL-18 [Debets *et al.* 2000] and this receptor subunit is present on Th1 cells but not Th2 cells, which instead express T1/ST2, a member of the IL-1R family [Löhning *et al.* 1998].

4.1.6.4 Signalling pathway of IL-18 receptor

The cytoplasmic domains of IL-1 and IL-18 receptors are homologous to Toll, a type I transmembrane receptor isolated from the *Drosophila* fly [O'Neill and Greene, 1998]. They are therefore both members of the IL-1R/toll-like receptor superfamily (IL-1R/TLR) [O'Neill and Dinarello, 2000]. The signalling pathway of IL-1 and IL-18 receptors involves recruitment of an adaptor molecule called MyD88, which initiates the IL-1 receptor-associated kinase pathway (IRAK) [Robinson *et al.* 1997; Takeuchi *et al.* 2000]. This pathway through tumour necrosis factor receptor-associated factor-6 (TRAF6) then leads to the activation of NF κ B, which is translocated to the nucleus [Robinson *et al.* 1997; Kojima *et al.* 1998] (figure 4.2).

Figure 4-2 IL-18 signalling pathway



4.1.7 Biological functions of IL-18

Studies of the biological effect of IL-18 have shown that the cytokine has multiple functions in the immune response. It induces IFN γ production from Th1 and NK cells [Okamura *et al.* 1995], particularly in combination with IL-12. However, as well as encouraging a Th1 biased response, under certain circumstances it can stimulate T and NK cells to produce Th2 cytokines.

4.1.7.1 Functions of IL-18 relating to Th1 response

IL-18 induces dose dependent proliferation of T cells, which is not inhibited by treatment with antibodies against IFN γ [Micallef *et al.* 1996]. IL-18 also synergises with IL-12 in the production of IFN γ from Th1 cells [Ahn *et al.* 1997]. In this study, in an IL-12 responsive T cell line, IL-18 stimulated only marginal IFN γ production in cell deprived of IL-12, but markedly enhanced production when both cytokines were present.

In contrast to IL-12, IL-18 alone is incapable of producing differentiation of naive T cells into Th1 cells. However, the presence of IL-18 accelerates IL-12-driven Th1 development [Robinson *et al.* 1997]. Also, mice deficient in IL-18 demonstrate fewer defects in Th1 function than IL-12-deficient mice [Takeda *et al.* 1998].

In addition to inducing IFN γ production, IL-18 also selectively enhances the Fas ligand-(FasL) mediated cytotoxicity of murine Th1 cells [Dao *et al.* 1996]. Fas ligand is expressed on activated or cytotoxic T cells and on engagement of this transmembrane protein with Fas on the target cell induces apoptosis. IL-18 does not enhance FasL on Th2 cells or naive T cells [Dao *et al.* 1996].

IL-18 acts as a costimulant with factors such as IL-2 and Con A to augment the production of IL-2 and GM-CSF from Th1 clones. This cytokine also induces proliferation of populations of Th1 clones. However, IL-18 induces little effect on the proliferation or cytokine production of Th2 cells [Micallef *et al.* 1996; Kohno *et al.* 1997].

4.1.7.2 Biological action of IL-18 on NK and CD8⁺ cells

As well as stimulating the cytotoxicity of Th1 cells, IL-18 also influences the cytotoxic effect of NK and CD8⁺ cells. These cells exert their cytotoxic function by apoptosis using perforin [Dao *et al.* 1998], FasL [Tsutsui *et al.* 1996] and TNF-related apoptosis-inducing ligand (TRAIL) [Wiley *et al.* 1995]. Cloned NK cells were shown to constitutively express FasL and kill target cells expressing Fas. This killing activity was upregulated by IL-18 but not IL-12 [Tsutsui *et al.* 1996]. On the other hand, IL-

18 does not to induce expression of TRAIL on activated NK cells [Kayagaki *et al.* 1999].

Experiments with IL-18 deficient mice showed that IL-18 is also important in the activity of NK cells. Impaired NK cell activity was demonstrated in both IL-18-deficient and IL-12-deficient mice. Administration of exogenous IL-12 or IL-18 restored NK cell activity in both IL-12 and IL-18 deficient mice which implies that each cytokine exerts its effect on NK cells independently of the other [Takeda *et al.* 1998].

4.1.7.3 IFN γ stimulation of other cells by IL-18

Cell types other than T and NK cells have been shown to respond to IL-18. Murine bone marrow-derived macrophages secrete high levels of IFN γ in response to IL-12 and IL-18, which neither cytokine stimulates alone [Munder *et al.* 1998]. IL-18 also augments the IFN γ production of murine splenic dendritic cells by IL-12 [Fukao *et al.* 2000] and microglia have been shown to express elements of the IL-18 receptor system and demonstrate expression of IRAK and TRAF 6 [Prinz and Hanisch, 1999].

Under certain circumstances B cells are able to respond to IL-18. When B cells are stimulated with anti-CD40 and IL-4 they produce IgG1 and IgE antibody. However when stimulated in this way in the presence of IL-12 and IL-18, there is inhibition of IgG1 and IgE with stimulation of IgG2a, and a rise in production of IFN γ [Yoshimoto *et al.* 1997].

4.1.7.4 Synergistic action of IL-18 and IL-12 on IFN γ production

IL-18 and IL-12 act synergistically to stimulate IFN γ production from T cells and NK cells [Ahn *et al.* 1997; Lauwerys *et al.* 1999]. This synergism occurs because each cytokine activates the IFN γ promoter by a separate pathway. The receptor for IL-12 activates STAT 4 [Jacobsen *et al.* 1995; Xu *et al.* 1996] as well as AP-1. The IFN γ promoter contains a binding site for both STAT 4 and AP-1, both of which are required for IL-12-dependent IFN γ promoter activity [Barbulescu *et al.* 1998]. IL-18

however functions via the IRAK-TRAF 6 pathway and the activation of NF- κ B [Robinson *et al.* 1997; Kojima *et al.* 1998].

These separate signalling pathways produce synergy of IL-12 and IL-18 in the induction of IFN γ transcription of T cells and this pathway functions independently of T cell receptor (TCR) signalling [Yang *et al.* 1999]. However anti-CD3 stimulation is required for the formation of Th1 cells from T cells in the presence of IL-12 and IL-18 showing that TCR is essential for T cell differentiation [Yoshimoto *et al.* 1998].

IL-12 and IL-18 have been shown to have a differential effect on CD8⁺ effector T cells. IL-12 alone was shown to decrease the CD8⁺/CD4⁺ ratios, IFN γ production and CTL activity of mixed lymphocyte cultures whereas IL-18 alone increased these levels but did not induce CD8⁺ effector cells. Together however, the two cytokines acted synergistically to produce CD8⁺ effector cells from mixed lymphocyte cultures [Okamoto *et al.* 1999]. This synergy has also been seen in the proliferation, activity and IFN γ production of NK cells [Tomura *et al.* 1998].

4.1.7.5 Induction of Th2 cytokines by IL-18

Although the stimulation of IFN γ is the main cytokine induced by IL-18 in the immune pathway, IL-18 can also stimulate Th2 cytokine expression by T and NK cells. IL-18 in combination with IL-2 induced strong production of IL-13 mRNA and protein from T and NK cells and enhanced the level of IL-13 induced by IL-2 alone [Hoshino *et al.* 1999]. This expression of IL-13 protein was increased in IFN γ knockout mice, implying that IFN γ plays a role in the regulation of IL-18-induced IL-13 production.

Also, naive CD4⁺ T cells treated with IL-18 and IL-2 showed TCR-independent production of IL-13, CD40L and IL-4 [Yoshimoto *et al.* 2000]. Stimulation of these cells with TCR stimulated further Th2 cytokine stimulation. Recent evidence shows that IL-18 is able to induce the development of Th2 cells from naive CD4⁺ T cells in the presence of IL-4, but these cells develop into Th1 cells if treated with anti-IL-4 antibody [Nakanishi *et al.* 2001b].

IL-18 in conjunction with IL-3 also induces expression of IL-18R α on basophils and mast cells and stimulates the production of IL-4 and IL-13 from basophils. This Th2 cytokine production is inhibited by treatment with a combination of IL-12 and IL-18 [Yoshimoto *et al.* 1999].

4.1.8 Role of IL-18 in defence against pathogens

The function of IL-18 in promoting cell-mediated immunity has led to studies of its role in defence against pathogens such as bacteria, viruses and fungal infection. One study showed that mice demonstrated protection against *Listeria monocytogenes* when injected with recombinant murine IL-18. Enhanced bacterial clearance occurred even when functional IFN γ was eliminated using an anti-IFN γ monoclonal antibody. The cytokine was shown to affect the innate immune response by production of TNF from macrophages, as well as stimulating antigen-specific T cells [Neighbors *et al.* 2001]. Caspase-1 deficient mice were shown to have impaired Th1 resistance to infection with *Candida albicans*. This corresponded with low IL-18, IL-12 and IFN γ levels. Th1 resistance was restored when mice were treated with exogenous IL-18 [Mencacci *et al.* 2000]. The role of IL-18 in anti-viral therapy has also been investigated. One study has shown that when recombinant murine IL-18 was administered to mice along with vaccinia virus, the clinical signs of disease were suppressed. This corresponded with an increase in NK and CTL activity. The beneficial effect was nullified by the use of anti-IFN γ antibody, showing that in this case, the role of IL-18 in IFN γ stimulation was obviously important [Tanaka-Kataoka *et al.* 1998].

4.1.8.1 Role of IL-18 in tumour therapy

The ability of IL-18 to promote NK, Th1, CD8⁺ cell cytotoxicity and T cell proliferation has been shown to be useful in stimulating a response against tumour antigens. Recombinant murine IL-18 suppressed over 75% primary tumour growth in murine T241 fibrosarcoma of SCID mice when administered intratumorally and systemically [Cao *et al.* 1999]. This study also showed that IL-18 suppressed tumour

angiogenesis shown by a significant decrease in blood vessel density in IL-18-treated mice compared to control animals.

In another experiment, a human parathyroid hormone leader sequence was fused to mature murine IL-18 and transfected into recombinant adenoviral vectors. Direct injection into established MCA205 murine fibrosarcoma cured all animals and produced protective immunity against the tumour. A synergistic effect was induced by simultaneous systemic IL-12 therapy with no side effects [Osaki *et al.* 1999]. The synergistic effect of IL-12 and IL-18 in tumour immunotherapy was also shown by the coadministration of murine IL-12, pro-IL-18 and caspase-1 cDNA by gene gun. This treatment suppressed the growth of murine mammary adenocarcinoma tumours implanted into the skin [Oshikawa *et al.* 1999].

4.1.8.2 Role of IL-18 as a vaccine adjuvant

Of particular relevance to this project is the great potential that IL-18 has shown as an adjuvant to vaccination. Several studies have been performed where IL-18 is delivered as an adjuvant in order to modulate the immune response towards cell mediated immunity. This shows particular promise with intracellular viruses such as HIV-1 where cell mediated immunity is important in viral clearance. In one study, the coinjection of IL-18 encoded DNA with an HIV-1 *nef* DNA prime/protein boost vaccine shifted the specific immune response towards Th1 cell mediated immunity [Billaut-Mulot *et al.* 2001a]. Similarly, the inclusion of murine plasmid IL-18 was used to modulate the response to a multiepitopic DNA vaccine for HIV-1. The vaccine constituted constructs encoding *gag*, *tat* and *nef* and the IL-18 gene decreased by 2 weeks the time taken to induce CTLs and increased the level of IL-2 and IFN γ induced by the antigen. Antibody titres against the viral proteins were decreased in this case [Billaut-Mulot *et al.* 2001b].

Studies have analysed the use of IL-18 as an adjuvant to DNA vaccines for feline retroviruses. A plasmid encoding feline mature IL-18 with an immunoglobulin secretory signal sequence derived from PsecI plasmid (Invitrogen Life Technologies), was used *in vivo* with an integrase deleted FIV DNA vaccine. The virus-specific CTL responses using IL-18 as an adjuvant were more consistent than using the DNA

vaccine alone. The efficacy of this vaccine was not improved by the inclusion of a plasmid encoding feline IL-12 [Dunham *et al.* 2002]. Of these cats, 33% demonstrated protection against FIV challenge.

The same IL-18 expression vector was used with an IL-12 plasmid as adjuvants to a DNA vaccine to FeLV. This combination produced complete protection against transient and persistent viraemia and 5 of 6 cats from latent infection [Hanlon *et al.* 2001]. Cats protected using these vaccines were shown to have higher FeLV-specific CTL responses in blood and lymphoid organs than control cats [Flynn *et al.* 2000a].

4.1.9 Design of IL-18 constructs

Experimental studies have used various forms of IL-18-encoding plasmid including pro-IL-18 cDNA alone [Oshikawa *et al.* 1999], pro-IL-18 coinoculated with IL-1 β converting enzyme (ICE) cDNA [Oshikawa *et al.* 1999] and mature-IL-18 cDNA [Giavedoni *et al.* 2001] (figure 4.3). On transcription and translation within the cell, both pro- and mature-IL-18 produced from these vectors pose problems in terms of secretion across the cell membrane.

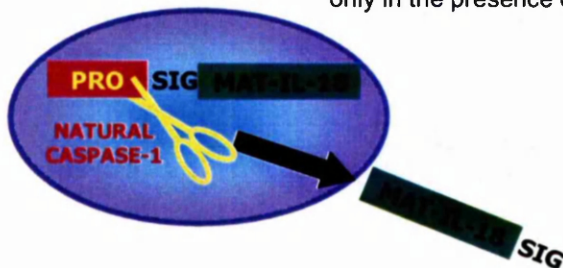
As mentioned previously, IL-18 is synthesised within the cell as pro-IL-18, a biological precursor molecule that lacks a signalling peptide. Pro-IL-18 requires cleavage by ICE or caspase-1 to produce the biologically active mature-IL-18, which contains the natural signal for secretion across the cell membrane [Okamura *et al.* 1995; Gu *et al.* 1997]. Cells transfected directly with pro-IL-18 must therefore rely on natural ICE present within the cell to allow secretion of bioactive mature-IL-18. Cells transfected with mature-IL-18 cDNA are unable to secrete IL-18 protein as they lack a natural signalling peptide. Therefore protein can only be released during apoptosis or cell injury. The coinoculation of pro-IL-18 and ICE cDNA partially addresses this problem [Oshikawa *et al.* 1999], but in this case the use of separate plasmids for each construct does not guarantee the production of both IL-18 and ICE protein in one cell.

For these reasons several groups have investigated the use of cDNA encoding signal peptides fused to the mature-IL-18 gene to encourage secretion from transfected cells.

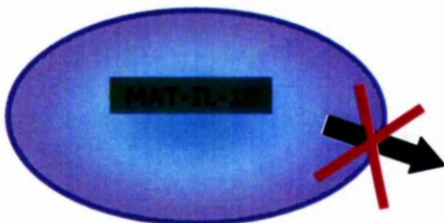
One study demonstrated antitumour activity of a vector encoding the prepro leader sequence of human parathyroid hormone fused to mature murine IL-18 [Osaki *et al.* 1999]. Others have shown resistance to *Mycobacterium avium* using murine mature-IL-18 fused to the human immunoglobulin kappa leader sequence [Kim *et al.* 2001].

Figure 4-3 IL-18 DNA vaccine constructs and their potential secretion

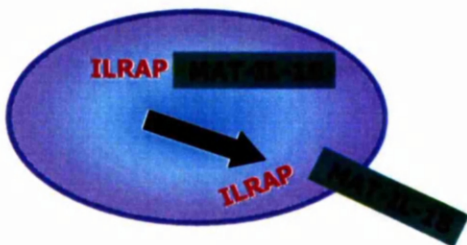
- 1) Inoculation of pro-IL-18 plasmid: secretion of mature-IL-18 with natural signalling peptide only in the presence of natural caspase-1



- 2) Inoculation of mature-IL-18 plasmid: no secretion of mature-IL-18 as no signalling peptide



- 3) ILRAP signal protein cleaved in the endoplasmic reticulum: secretion of mature IL-18



4.1.10 *In vitro* measurement of bioactivity of IL-18

Since its isolation, the standard assay used to measure the *in vitro* bioactivity of IL-18 has been the production of IFN γ from fresh PBMC populations [Ushio *et al.* 1996]. These cells were cultured in the presence of IL-18 samples and the supernatants were measured for human IFN γ production by specific ELISA. This system presents

several disadvantages. Firstly the preparation of PBMC cells is laborious and cell responses vary between blood donors. In addition, PBMC cells under certain conditions are able to produce IFN γ in response to other cytokines such as IL-12, which decreases the specificity of the assay [McDyer *et al.* 1998].

These disadvantages led to the development of a new bioassay based on IFN γ production from KG-1 cells. This is a human myelomonocytic cell line that produces human IFN γ (hIFN γ) in response to IL-18 without the requirement of a costimulatory signal [Konishi *et al.* 1997]. A study of this cell line showed that an NF- κ B binding site was stimulated at the regulatory region of the IFN γ gene in response to human IL-18 [Kojima *et al.* 1999]. Konishi *et al.* developed this bioassay by incubating test samples in a KG-1 cell suspension of 3×10^6 cells/ml for 24 hours. The level of hIFN γ produced from samples and human IFN γ standards was measured using a specific ELISA. Human IL-18 was detected in a dose dependent manner, which was suppressed by the addition of human serum. Background levels of hIFN γ were produced when cells were incubated with human IL-12, confirming the specificity of this assay to IL-18.

Konishi *et al.* demonstrated detection of murine IL-18 using this assay, but at 100-fold lower sensitivity. With the aim of improving the sensitivity to murine protein, they produced a stable cell line of KG-1 cells expressing murine IL-18 receptor. This modified cell line was shown to express large amounts of hIFN γ in response to both human and murine IL-18 in a dose dependent manner, increasing significantly the sensitivity of this assay to murine IL-18 [Taniguchi *et al.* 1998].

4.1.11 Aim of work

The aim of this work was to analyse the level of expression and bioactivity of several feline IL-18 constructs *in vitro*. A feline signal IL-18 construct, PsecI-IL-18 was cloned previously using a PsecI plasmid, a vector derived from pCI-neo (Promega) containing a synthetic immunoglobulin secretory component [Hanlon, 1999]. This construct was used *in vivo* with IL-12 as a vaccine adjuvant to a DNA vaccine for FeLV [Hanlon *et al.* 2001] and a DNA vaccine to FIV [Dunham *et al.* 2002].

However, the *in vitro* expression and bioactivity of PsecI-IL-18 had not been confirmed.

This work involved the development of a new feline signal IL-18, ILRAP-IL-18. This construct consisted of feline mature-IL-18 fused to the human signal sequence for IL-1 β receptor antagonist protein (ILRAP) [Wingren *et al.* 1996]. *In vitro* experiments demonstrated protein expression and a KG-1 bioassay was developed to detect feline IL-18 protein *in vitro*. This system was used to measure the bioactivity of pro-, mature-, PsecI- and ILRAP-IL-18 supernatants and cell lysates from cell transfections. In addition the feline IL-18R α was cloned. This receptor may be used to produce a stable cell line of KG-1 cells to further increase the sensitivity of the KG-1 assay to feline IL-18.

4.2 MATERIALS AND METHODS

4.2.1 Cloning of feline pro-, mature- and PsecI-IL-18 constructs

Feline pro-IL-18 and mature-IL-18 were a kind donation from Dr Linda Hanlon (Molecular Oncology Laboratories, University of Glasgow). cDNA encoding pro-IL-18 was isolated by RT-PCR of mRNA from LPS-stimulated feline alveolar macrophages [Hanlon, 1999]. This fragment was cloned into pCI-neo expression vector (Promega).

Mature-IL-18 was amplified from the pro-IL-18/pCI-neo construct by PCR using specific primers and also cloned into pCI-neo expression vector [Hanlon, 1999].

L. Hanlon performed the cloning of PsecI-IL-18. Specific primers were designed to amplify mature-IL-18 with restriction sites encoded at the 5' and 3' end. These sites were used to clone the mature-IL-18 fragment into PsecI expression vector [Hanlon, 1999].

4.2.1.1 Cloning of feline ILRAP-IL-18 construct

ILRAP-IL-18 was cloned by L. McMonagle, Retrovirus Research Laboratories, University of Glasgow. The DNA template used for PCR amplification was feline mature-IL-18 [Hanlon, 1999]. Two sets of specific primers were designed to amplify mature-IL-18 with ILRAP-encoding sequence at the 5' end of the fragment. The primary PCR used the following primers with *Taq* DNA polymerase:

Primary 5' primer: 5' -CGC AGT CAC CTA ATC ACT CTC CTC CTC TTC CTG TTC CTG TTC CAT TCA GAG ACG ATC TGC TAC TTT GGC AAG CTT G -3'

Primary 3' primer: 5' -GCG CGT CGA CCT AAT TCT TGT TTT GAA CAG TG- 3'

This 5' primer encoded half of the ILRAP sequence followed by feline mature-IL-18 sequence. The 3' primer encoded the 3' feline mature-IL-18 sequence. A secondary

PCR was used to amplify a full length ILRAP-IL-18 fragment from the 1^o PCR. The same 3' primer was used in combination with the following 5' primer:

Secondary 5' primer: 5' - GGC AGA ATT CGC CAT GAA ATC TGC AGA GGC CTC CGC AGT CAC CTA ATC AC - 3'

This produced a full length ILRAP-IL-18 construct which was cloned into pCR3.1 vector (Invitrogen Life Technologies). The fragment was excised from pCR3.1 using an *EcoR* I/ *Sal* I restriction digest and cloned into similarly digested pCI-neo expression vector (Promega).

4.2.2 Transfection of IL-18 constructs into CHO cell line

DNA for transfection of pro-IL-18, mature-IL-18, ILRAP-IL-18 and pCI-neo expression vector was prepared using Qiagen plasmid Mega Kit (Qiagen) and stored at -20°C until further use. CHO cells were maintained in 175 cm² culture flasks and 20 µg of DNA was used for each transfection. All transfections were carried out under the same conditions using LipofectAMINE Reagent (Invitrogen Life Technologies) according to the instructions of the manufacturer. The transfections were incubated for 48 hours in an incubator at 37°C with 5% CO₂.

In a separate experiment, transfections of feline PsecI-IL-18, ILRAP-IL-18 and pCI-neo vector were performed in parallel, in order to compare the levels of protein expression and secretion of feline signal-IL-18 constructs.

The supernatants and cell lysates were then harvested for analysis. The supernatant was removed from each transfection and any cellular debris eliminated by centrifugation at 1000 rpm for 5 minutes. The supernatant was then aliquoted and stored at -20°C until further use. The cells were detached from each flask using a cell scraper and the cells from each transfection were harvested in the appropriate buffer amounts. These amounts were based on observed expression levels between the different IL-18 constructs; constructs eliciting the highest protein expression were diluted in larger buffer volumes. As a result, cells from the pro-IL-18 transfection

were harvested in separate 4 ml PBS and 4 ml caspase buffer (CB) aliquots, mature-IL-18 in 4 ml PBS, ILRAP-IL-18 and PsecI-IL-18 in 2 ml PBS and pCI-neo in 1 ml PBS and 1 ml CB. Pro-IL-18 caspase buffer cell lysate was included in order to allow digestion of pro-IL-18 with caspase-1 to produce bioactive mature-IL-18. These cells were then frozen and thawed three times in a dry ice ethanol bath and 37°C water bath respectively, in order to rupture cell membranes. Cell lysate debris was pelleted by centrifugation at 14000 rpm for 30 seconds and the supernatant was aliquoted and stored at -20°C until later use. Prior to storage, pro-IL-18 cell lysate was digested to produce mature-IL-18 as described below.

4.2.2.1 Preparation of bioactive mature-IL-18 from pro-IL-18 cell lysate

Pro-IL-18 cell lysate in caspase buffer produced from CHO cell transfections was incubated overnight at room temperature with human recombinant caspase-1 (Calbiochem, UK) at a concentration of 2500 U/ml. This sample was then stored at -20°C until further use.

4.2.3 *In vitro* expression of IL-18 protein products using Western blot analysis

Expression of pro-IL-18, mature-IL-18, ILRAP-IL-18 and pCI-neo transfection products were analysed using SDS-PAGE and immunodetection. The primary antibody was a sheep anti-feline IL-18 polyclonal antibody, a kind donation from Dr Norman Flynn (Retrovirus Research Laboratories, University of Glasgow), used at a 1:2000 dilution. The secondary antibody was anti-sheep IgG peroxidase conjugate, delivered at a dilution of 1:2000.

Before separation on a gel, each IL-18 cell lysate sample was diluted appropriately in dH₂O in order to balance the protein level in each sample. A volume of 20 µl of undiluted supernatant sample with 5 µl protein loading buffer was used per well. The protein samples were separated on a 15% SDS-PAGE gel under denaturing conditions as outlined in 2.2.10. In addition, 3.2 ng of recombinant equine IL-18 was run

alongside as a positive control. The gel was transferred to a PVDF membrane and probed as described in 2.2.11 using the antibody concentrations described above.

Samples of feline ILRAP-IL-18, PsecI-IL-18 and pCI-neo cell lysate and supernatant were also compared on an SDS-PAGE gel prepared as described previously. 18 µl of sample was used in each well with 5 µl protein loading buffer. This gel was to allow comparison of protein expression and secretion of signal-IL-18 constructs. Equine ILRAP and PsecI-IL-18 constructs were also analysed by Western blot analysis. All samples were exposed to a primary antibody of rabbit anti-equine polyclonal antibody (prepared in our laboratories) at a concentration of 1:4000 and a secondary antibody of anti-rabbit IgG peroxidase conjugate at a dilution of 1:2000. This rabbit anti-equine polyclonal antibody was found to be cross-reactive with feline IL-18 protein.

4.2.4 *In vitro* bioactivity of IL-18 protein products

The bioactivity of caspase-1 digested pro-IL-18, mature-IL-18, ILRAP-IL-18, PsecI-IL-18 and pCI-neo transfection products were analysed using a KG-1 bioassay system, which measures the level of hIFN γ production by this cell line in response to IL-18. [Konishi *et al.* 1997].

4.2.4.1 IL-18 bioassay samples

Of the supernatant samples, neither pro-IL-18 nor mature-IL-18 displayed detectable levels of protein secretion from the cell in either Western blot or previous bioassays and were therefore excluded from this experiment. However comparison of ILRAP-IL-18 and PsecI-IL-18 supernatants was performed along with pCI-neo supernatant as a negative control.

The following cell lysate samples diluted in PBS were used: pro-IL-18, mature-IL-18, ILRAP-IL-18, PsecI-IL-18 and pCI-neo as a negative control. Lysate samples diluted in caspase buffer were prepared as follows: pro-IL-18 digested with caspase and pCI-neo digested with caspase as a negative control; caspase-digested pro-IL-18 with sheep anti-feline IL-18 polyclonal antibody (34ng/µl) and caspase digested pCI-neo

also with antibody as a negative control. In addition a negative control for the antibody itself was included by using caspase-digested pro-IL-18 with FeLV rabbit polyclonal antibody at a similar concentration. The inclusion of an IL-18 specific neutralising antibody allows binding and inactivation of the bioactive IL-18 molecule verifying specificity of the hIFN γ production to IL-18 protein.

4.2.4.2 IL-18 bioassay

A 1:2 titration of each sample was made in KG-1 medium (Iscove's Modified Dulbecco's medium, 20% FCS, 1 x Penicillin/Streptomycin and 2 mM L-Glutamine) and 150 μ l of each dilution was added to a flat-bottom 96 well plate. The higher protein expression of pro-IL-18 transfection required larger dilution factors than either mature-IL-18 or ILRAP-IL-18. As a result, titrations from 1:1250 to 1:20000 were used for pro-IL-18 and 1:32 to 1: 1024 were used for mature-IL-18, ILRAP-IL-18 and PsecI-IL-18. This plate was then incubated at 4°C for 24 hours on a shaking platform.

After 24 hours, 100 μ l of each sample was aliquoted onto a fresh 96 well plate. KG-1 cells were suspended at a cell density of 3×10^6 cells/ml in fresh culture medium and human recombinant TNF α was added to a concentration of 10 ng/ml. 100 μ l (3×10^5 cells) was added to each protein sample and mixed gently. The plate was then incubated for 24 hours at 37°C with 5% CO $_2$.

The cells were pelleted by centrifugation of the plate at 1000 rpm for 5 minutes and the amount of IFN γ in the supernatant was measured using the Quantikine human IFN γ kit (R + D Systems) under the manufacturer's instructions. This system consists of a quantitative sandwich enzyme immunoassay using an ELISA plate pre-coated with an IFN γ -specific polyclonal antibody. 100 μ l sample was added to 100 μ l assay diluent and incubated for 2 hours at room temperature. Standard samples of human IFN γ were completed alongside at concentrations of 1000-15.6 pg/ml. Any hIFN γ present in the transfection sample became bound to the plate and unbound substances were removed by washing the plate 4 times in wash buffer. Each well was then incubated for 2 hours with 200 μ l conjugate containing an anti-IFN γ polyclonal antibody conjugated to horseradish peroxidase. The washing step was repeated and a substrate

was added to each well, which develops colour in proportion to the level of IFN γ bound to each well. The substrate was incubated on the plate in the dark for 30 minutes at room temperature. The colour development was stopped by adding 50 μ l of stop solution, and the intensity of colour measured using a spectrophotometer at 560 nm.

4.2.5 Cloning of feline IL-18 receptor

4.2.5.1 Design of primers

Primers for the amplification of ligand binding subunit of the feline IL-18 receptor (FIL-18R α) were designed using a consensus sequence of human IL-18R α [Torigoe *et al.* 1997] and murine IL-18R α [Taniguchi *et al.* 1998]. Focus was placed on areas of sequence which would require primers of low degeneracy. This involved locating blocks of amino acids that were conserved between species. In addition, low degeneracy primers were favoured in areas of amino acids encoded by only one possible codon sequence rather than several codons. The sequence of oligonucleotides tended to be based on the human IL-18R α sequence because in general, human sequences display greater homology with feline than murine sequences. All primer positions are demonstrated in figure 4.4.

An initial set of primers was designed to amplify preliminary fragments of IL-18R α . The sequence data produced from these fragments facilitated the design of subsequent primers internal to the known sequence and at the start and stop codon, again using the published human receptor sequence as reference. These primers were then used in combination to amplify the fragments upstream and downstream from the original isolated fragments to obtain the full receptor sequence.

Primer	Sequence (5'-3')
Initial primers	
F9a	CCTCATTGTATAAARAAYTGCAA
F9b	CCTCATTGTATAAARAAYTGTA
F9s	ACATCATTGTATAAGAAGCTG
R6	GTAAAGAAGGTTYTTCCARAA
R8	CTGGCCACAGTRCARTTRTA
R8s	TCCCGTGCTGGCCACAGTGC
Subsequent primers	
F1d	GCGAAGCAGAATCCAAANNATG
R10	AATATCCCTGATCTTCAAAGCTC
F11	CTTGAAAGTGGACTCCATGAAGC
R12	TTAAGACTCGGAAAGAAGACAG

N = A/G/C/T H = A/C/T R = A/G Y = C/T

Fragments attained from these reactions resulted in a full preliminary FIL-18R α sequence with a small area of primer-derived sequence at both 5' and 3' ends. Primers F1d and R12 were then modified and extended using exact FIL-18R α sequence to produce 3DN and 12NL respectively. These were used in combination with both existing primers and new primers, F13 and F15 derived from human and equine IL-18R α sequence, to amplify more fragments. Additional sequence information was gained by using F9SF which was a primer based on F9s using exact feline IL-18 receptor sequence, and R16SF and R17SF which were reverse primers of exact feline sequence situated close to 12NL primer.

Feline IL-18R α primers

Name of primer	Sequence (5'-3')
3DN	ATGCGTCATATAGAACTGCTCTTAACG
12NL	GTTAAGACTCGGAAAGAAGCAGGAAAGACTTCAGATT
F13	GTGTTGGAGAAACATTTTGG
F15	ATCCACTCACTGATAGAGAA
9SF	ACTTCACTGTATAAGAAGCTG
R16SF	GCAGGCATCAGGTACAGAAG
R17SF	CAGATTCAGTTCCACGAGGC

4.2.5.2 Isolation of MYA-1 cell RNA

The MYA-1 cell line was maintained as described in 2.2.15.1. Total RNA was obtained from 2×10^7 cell aliquots using the Gentra Purescript RNA isolation kit (Flowgen) according to the manufacturer's instructions. This kit lyses cells in the presence of an RNA preservative using an anionic detergent which solubilises the cellular components. The RNA preservative limits the activity of RNases that are present in the cell and elsewhere in the environment. The contaminating DNA and proteins are then removed by salt precipitation.

RNA from each cell aliquot was resuspended in 200 μ l DEPC-treated water and treated with DNase. Purescript DNase I (Gentra systems) was used with 1 x reaction buffer at a concentration of 0.017 U/ μ l. This reaction was incubated at 37°C for 1 hour and the DNase was inactivated at 65°C for 10 minutes. The RNA was aliquoted and stored at -20°C until further use.

4.2.5.3 Reverse transcription-PCR of feline IL-18R α

Total RNA was reverse transcribed and PCR fragments amplified using the Access RT-PCR System (Promega). Basic reaction concentrations and conditions are described in 2.2.13. Annealing temperatures were selected according to the melting temperatures of each primer combination used. The use of PCR Express machines (Hybaid Limited) allowed a range of annealing temperatures to be used simultaneously, permitting efficient identification of optimal PCR conditions.

Fragments of expected sizes were cloned into pCR-2.1-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen Life Technologies) under the instructions of the manufacturer. TOPO cloning reactions were transformed into One Shot Chemically Competent *E. coli* (Invitrogen Life Technologies) and minipreparations of plasmid DNA made from the colonies produced. Plasmid DNA was digested with appropriate enzymes within the cloning vector to excise the insert. These fragments were visualised on a 0.8% agarose gel stained with ethidium bromide.

Confirmation of positive clones was carried out by sequencing using the ABI Prism system. Forward and reverse primers featured in the cloning vector were used to verify both forward and reverse sequence. This data was analysed using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin), and by visualisation of the chromatogram. A consensus nucleotide sequence of feline IL-18R α was produced which was extrapolated to establish the amino acid sequence of the receptor.

Figure 4-4 Consensus sequence of human and murine IL-18 α

Key

Red text	-initial set of primers
Blue text	-subsequent primers
Green text	-modified primers
Purple text	-degenerate primers derived from equine and human IL-18R α sequence

Degeneracy sequences

R - A/G

Y - C/T

N - A/G/C/T

H - A/C/T

```

                                F3DN ATGCG TCATATAGAA
                                F1d  GCGA AGCAGAATCC AAANNATG
hill18r  ~~~~~ ~GCCATTTGA AGCAGAATCC AAACCATGAA TTGTAGAGAA
mil18r   AAACAAGAGA TACCATTCAA AGTGGAAGCC TAAACATGCA TCATGAAGAA
Con      ----- --CCATT--A AG--GAA-CC -AA-CATG-A T--T--AGAA
                                CTGCTCTTAA CG

hill18r  TTACCCTTGA CCCTTTGGGT GCTTATATCT GTAAGCACTG CAGAATCTTG
mil18r   TTAATCTTGA CACTCTGCAT TCTCATGTGT AAAAGTGCCT CAAAAAGTTG
Con      TTA--CTTGA C-CT-TG--T -CT-AT---T --AAG--C-- CA-AA--TTG

                                101                                150
hill18r  TACTTCACGT CCCACATTA CTGTGGTTGA AGGGGAACCT TTCTATCTGA
mil18r   TATTCAACGA TCACAAATTC ATGTGGTAGA GGGAGAACCT TTTTATCTGA
Con      TA-T---CG- -C-CA-ATT- -TGTGGT-GA -GG-GAACCT TT-TATCTGA

                                151                                200
hill18r  A...ACATTG CTCGTGTTCA CTTGCACATG AGATTGAAAC AACCACCAAA
mil18r   AGCCATGTGG CATATCTGCA CCAGTGCACA GGAATGAAAC AGCCACCATG
Con      A---A--T-G C---T-T-CA C--G--CA-- -GA-TGAAAC A-CCACCA--

                                201                                250
hill18r  AGCTGGTACA AAAGCAGTGG ATCACAGGAA CATGTGGAGC TGAACCCAAG
mil18r   AGATGGTTCA AAGGCAGTGC TTCACATGAG TATAGAGAGC TGAACAACAG
Con      AG-TGGT-CA AA-GCAGTG- -TCACA-GA- -AT---GAGC TGAAC---AG

```

251 300

hil18r GAGTTCCTCG AGAATTGCTT TGCATGATTG TGTTTGGAG TTTTGGCCAG
mil18r AAGCTCGCCC AGAGTCACTT TTCATGATCA CACCTTGGAA TTCTGGCCAG
Con -AG-TC--C- AGA-T--CTT T-CATGAT-- ----TTGGA- TT-TGGCCAG

301 350

hil18r TTGAGTTGAA TGACACAGGA TCTTACTTTT TCCAAATGAA AAATTATACT
mil18r TTGAGATGGA GGATGAGGGA ACGTACATTT CTCAAGTCGG AAATGATCGT
Con TTGAG-TG-A -GA----GGA -C-TAC-TTT --CAA-T--- AAAT-AT--T

351 400

hil18r CAGAAATGGA AATTAAATGT CATCAGAAGA AATAAACACA GCTGTTTCAC
mil18r CGCAATTGGA CCTTAAATGT CACCAAAAGA AACAAACACA GCTGTTTCTC
Con C--AA-TGGA --TAAATGT CA-CA-AAGA AA-AAACACA GCTGTTTC-C

401 450

hil18r TGAAAGACAA GTAAC TAGTA AAATTGTGGA AGTTAAAAA TTTTTCAGA
mil18r TGACAAGCTC GTGACAAGCA GAGATGTTGA AGTTAACAAA TCTCTGCATA
Con TGA-A--C-- GT-AC-AG-A -A--TGT-GA AGTTAA-AAA T-T-T-CA-A

F9b CCTCATTG
F9s F9a CCTCATTG

hil18r TAACCTGTGA AAACAGTTAC TATCAAACAC TGGTCAACAG CACATCATTG
mil18r TCACTGTGTA GAATCCTAAC TATGAAGAGC TGATCCAGGA CACATGGCTG
Con T-AC-TGT-A -AA---T-AC TAT-AA---C TG-TC-A--- CACAT---TG
TATAAARAAYT GTAA
TATAAARAAYT GCAA

hil18r TATAAGAAGCT GTAAAAAGCT ACTACTGGAG AACAATAAAA ACCCAACGAT
mil18r TATAAGAAGCT GTAA..... .GGAA ATATCCAAAA CCCCAAGGAT
Con TATAAGAAGCT GTAA----- -GGG- A-----AAAA -CCCAA-GAT

551 R10 600

hil18r AAAGAAGAAC GCCGAGTTTG AAGATCAGGG GTATTACTCC TGC GTGCATT
mil18r CCTGAAGGAT GCCGAGTTTG GAGATGAGGG CTACTACTCC TGC GTGTTTT
Con ---GAAG-A- GCCGAGTTTG -AGAT-AGGG -TA-TACTCC TGC GTG--TT

601 650

hil18r TCCTTCATCA TAATGGAAAA CTATTTAATA TCACCAAAAC CTTCAATATA
mil18r CTGTCCACCA TAATGGGACA CGGTACAACA TCACCAAGAC TGTCAATATA
Con ---T-CA-CA TAATGG-A-A C--T--AA-A TCACCA-AAC --TCAATATA

651 700

hil18r ACAATAGTGG AAGATCGCAG TAATATAGTT CCGGTCTTC TTGGACCAAA
mil18r ACAGTTATTG AAGGAAGGAG TAAAGTAACT CCAGCTATTT TAGGACCAAA
Con ACA-T--T-G AAG---G-AG TAA--TA--T CC-G-T-TT T-GGACCAAA

701 750

hil18r GCTTAACCAT GTTGCAGTGG AATTAGGAAA AAACGTAAGG CTCAACTGCT
mil18r GTGTGAGAAG GTTGGTGTAG AACTAGGAAA GGATGTGGAG TTGAACTGCA
Con G--T-A--A GTTG--GT-G AA-TAGGAAA --A-GT---G -T-AACTGC-

751 800

hil18r CTGCTTTGCT GAATGAAGAG GATGTAATTT ATTGGATGTT CGGGGAAGAA
mil18r GTGCTTCATT GAATAAAGAC GATCTGTTTT ATTGGAGCAT CAGGAAAGAG
Con -TGCTT---T GAAT-AAGA- GAT-T--TTT ATTGGA---T C-GG-AAGA-

801 850

hil18r AATGGATCGG ATCCTAATAT ACATGAAGA. .GAAAGAAA TGAGAATTAT
mil18r GACAGCTCAG ACCCTAATGT GCAAGAAGAC AGGAAGGAGA CGACAACATG
Con -A--G-TC-G A-CCTAAT-T -CA-GAAGA- --GAA-GA-A -GA-AA----

851 900

hil18r GACTCCAGAA GGCAAAATGGC ATGCTTCAAA AGTATTGAGA ATTGAAAATA
mil18r GATTTCTGAA GGCAAACTGC ATGCTTCAAA AATACTGAGA TTTCAGAAAA
Con GA-T-C-GAA GGCAAA--GC ATGCTTCAAA A-TA-TGAGA -TT-A-AA-A

R8 **TARA ARTGRACGTG GGCCAG** R8s

hil18r TTGGTGAAAG CAATCTAAAT GTTTTATATA ATT**GCACGTG GGCCAGCACG**
mil18r TTACTGAAAA CTATCTCAAT GTTTTATATA ATTGCACCGT GGCCAACGAA
Con TT--TGAAA- C-ATCT-AAT GTTTTATATA ATTGCAC-GT GGCCA-C---

951 1000

hil18r **GGAGGCACAG** ACACCAAAAG CTTCATCTTG GTGAGAAAAG CAGACATGGC
mil18r GAAGCCATAG ACACCAAGAG CTTCGTCTTG GTGAGAAA. .AGAAATACC
Con G-AG-CA-AG ACACCAA-AG CTTC-TCTTG GTGAGAAA-- -AGA-AT--C

1001 1050

hil18r TGATATCCCA GGCCACGTCT TCACAAGAGG AATGATCATA GCTGTTTTGA
mil18r TGATATCCCA GGCCATGTCT TTACAGGAGG AGTAACTGTG CTTGTTCTCG
Con TGATATCCCA GGCCA-GTCT T-ACA-GAGG A-T-A---T- --TGTT-T--

1051 1100

hil18r TCTTGGTGGC AGTAGTGTGC CTAGTGACTG TGTGTGTCAT TTATAGAGTT
mil18r CCTCTGTGGC AGCAGTGTGT ATAGTGATTT TGTGTGTCAT TTATAAAGTT
Con -CT--GTGGC AG-AGTGTG- -TAGTGA-T- TGTGTGTCAT TTATA-AGTT

1101 1150

hil18r GACTTGGTTC TATTTTATAG ACATTTAACG AGAAGAGATG AAACATTAAC
mil18r GACTTGGTTC TGTCTATAG GCGCATAGCG GAAAGAGACG AGACACTAAC
Con GACTTGGTTC T-TT-TATAG -C---TA-CG --AAGAGA-G A-ACA-TAAC

1151 1200

hil18r AGATGGAAAA ACATATGATG CTTTGTGTC TTACCTAAAA GAATGCCGAC
mil18r AGATGGTAA ACATATGATG CCTTGTGTC TTACCTGAAA GAGTGTCTATC
Con AGATGG-AAA ACATATGATG C-TTGTGTC TTACCT-AAA GA-TG-C-C

F13 **GTG**

hil18r CTGAAAAATGG AGAGGAGCAC ACCTTTGCTG TGGAGATTTT GCCCAGGGTG
mil18r CTGAGAATAA AGAAGAGTAT ACTTTTGCTG TGGAGACGTT ACCCAGGGTC
Con CTGA-AAT-- AGA-GAG-A- AC-TTGTCTG TGGAGA--TT -CCCAGGGT-

TTGGAGAABC ADTTDGG

hil18r TTGGAGAAAC ATTTTGGGTA TAAGTTATGC ATATTTGAAA GGGATGTAGT
mil18r CTGGAGAAAC AGTTTGGGTA TAAGTTATGC ATATTTGAAA GAGATGTGGT
Con -TGGAGAAAC A-TTTGGGTA TAAGTTATGC ATATTTGAAA G-GATGT-GT

F15 ATCCA CTCACTGATH GABAA

hil18r GCCTGGAGGA GCTGTTGTTG ATGAAATCCA CTCACTGATA GAGAAAAGCC
mil18r GCCTGGCGGA GCTGTTGTCG AGGAGATCCA TTCACTGATA GAGAAAAGCC
Con GCCTGG-GGA GCTGTTGT-G A-GA-ATCCA -TCACTGATA GAGAAAAGCC

1351 1400

hil18r GAAGACTAAT CATTGTCCTA AGTAAAAGTT ATATGTCCTA TGAGGTCAGG
mil18r GGAGGCTAAT CATCGTTCTC AGCCAGAGTT ACCTGACTAA CGGAGCCAGG
Con G-AG-CTAAT CAT-GT-CT- AG--A-AGTT A--TG-CTAA -G--G-CAGG

F11 GCCTTG AAAGTGGACT CCATGAAG

hil18r TATGAACTTG AAAGTGGACT CCATGAAGCA TTGGTGGAAG GAAAAATTAA
mil18r CGTGAGCTCG AGAGTGGACT CCACGAAGCA CTGGTAGAGA GGAAGATTAA
Con --TGA-CT-G A-AGTGGACT CCA-GAAGCA -TGGT-GA-A G-AA-ATTAA

1451 1500

hil18r AATAATCTTA ATTGAATTTA CACCTGTTAC TGACTTCACA TTCTTGCCCC
mil18r GATCATCTTA ATTGAGTTTA CTCCAGCCAG CAACATCACC TTTCTCCCCC
Con -AT-ATCTTA ATTGA-TTTA C-CC-G--A- --AC-TCAC- TT--T-CCCC

1501 1550

hil18r AATCACTAAA GCTTTTGAAA TCTCACAGAG TTCTGAAGTG GAAGGCCGAT
mil18r CGTCGCTGAA ACTCCTGAAG TCCTACAGAG TTCTAAAATG GAGGGCTGAC
Con --TC-CT-AA -CT--TGAA- TC--ACAGAG TTCT-AA-TG GA-GGC-GA-

R6 TTRTGG AAYAACCTTC TTTAC

hil18r AAATCTCTTT CTTATAACTC AAGGTTCTGG AAGAACCTTC TTTACTTAAT
mil18r A...GTCCCT CCATGAACTC AAGGTTCTGG AAGAATCTTG TTTACCTGAT
Con A----TC--T C----AACTC AAGGTTCTGG AAGAA-CTT- TTTAC-T-AT

R12NL **GAATCTGAA GTCTTTCCTG**

R12 **CTG**

hil18r GCCTGCAAAA ACAGTCAAGC CAGGTAGAGA CGAACCGGAA GTCTTGCCCTG
mil18r GCCCGCAAAA GCCGTCAAGC CATGGAGAGA GGAGTCGGAG GCGCGGTCTG
Con GCC-GCAAAA -C-GTCAAGC CA-G-AGAGA -GA--CGGA- G----G-CTG

TTCTTTCCGAGTCTTAAC

TTCTTTCCGA GTCTTAACGC

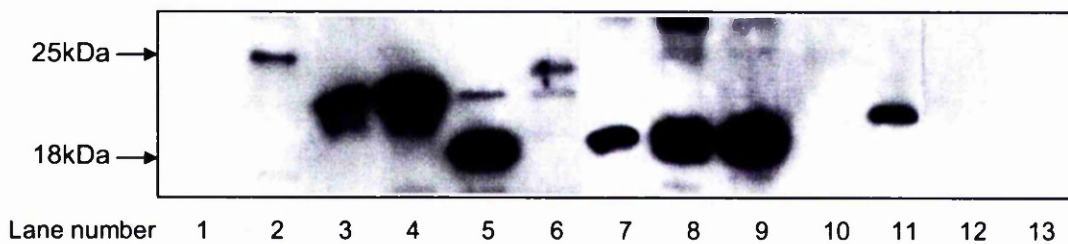
hil18r TTCTTTCCGA GTCTTAATCT TCAGAAACAG TGAACGCCAA AAAGAAGTCA
mil18r TTCTTCTCAGC ACCTTGAGCT CCAGACGAGC TTGATGTCAA AAGCAAGTGA
Con TTCT-TC-G- --CTT-A-CT -CAGA----- T--A-G-CAA AA--AA-T-A

4.3 RESULTS

4.3.1 *In vitro* expression of feline IL-18 protein products

Feline pro-IL-18, mature-IL-18, ILRAP-IL-18, and pCI-neo were transfected into CHO cells and supernatants and cell lysates harvested. A 15% SDS-PAGE gel was prepared using various dilutions of the feline IL-18 protein constructs (figure 4.5).

Figure 4-5 SDS-PAGE gel of feline pro-, mature- and ILRAP-IL-18 protein



This is an autoradiograph of a 15% SDS-PAGE gel showing feline IL-18 protein constructs.

Lanes are as follows: **lane 1:** kaleidoscope protein marker, **lane 2:** Novagen Perfect Protein marker. **CELL LYSATES:** **lane 3:** pro-IL-18 in PBS (1:40), **lane 4:** pro-IL-18 in caspase buffer (1:40), **lane 5:** pro-IL18 caspase buffer digested with human recombinant caspase-1 (1:40), **lane 7:** ILRAP-IL-18, **lane 9:** mature-IL-18 (1:2), **lane 12:** pCI-neo. **SUPERNATANTS:** **lane 6:** pro-IL-18, **lane 8:** ILRAP-IL-18, **lane 10:** mature-IL-18, **lane 13:** pCI-neo, **lane 11:** recombinant bacterial equine IL-18 (1.6ng).

The Western blot demonstrates expression of uncleaved pro-IL-18 protein 24 kDa in size (lanes 3 and 4), and mature cleaved IL-18 protein, 18 kDa in size (lane 5). In addition, 18 kDa recombinant equine IL-18 protein was used as a positive control (lane 11).

IL-18 was detected in cell lysates of undigested and digested pro-IL-18, mature-IL-18 and ILRAP-IL-18 transfections. In order to load equivalent amounts of protein, each cell lysate was diluted appropriately prior to running on the gel. From these dilutions it can be estimated that expression of pro-IL-18 is approximately 40-fold greater than ILRAP-IL-18 and mature-IL-18 is approximately twice that of ILRAP-IL-18 protein expression.

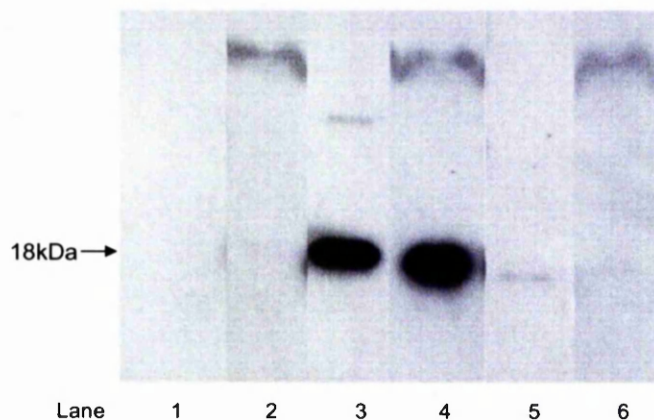
Pro-IL-18 and mature-IL-18 were not detected in transfection supernatants (lanes 6 and 10 respectively). In contrast, ILRAP-IL-18 was detected in the supernatant (lane 8). This suggests that detectable secretion of IL-18 protein occurs in cells transfected with the ILRAP-IL-18 construct but not in cells transfected with pro- or mature-IL-18.

4.3.2 *In vitro* expression of ILRAP-IL-18 and PsecI-IL-18

Parallel transfections of feline ILRAP-IL-18, PsecI-IL-18 and pCI-neo constructs were performed and cell lysates and supernatants were run on a Western blot (figure 4.6). ILRAP-IL-18 protein is clearly detected both in the cell lysate and in the transfection supernatant as shown by the large 18 kDa band. A much fainter, similarly sized band can be visualised in the PsecI-IL-18 cell lysate, but no band is visible in the transfection supernatant.

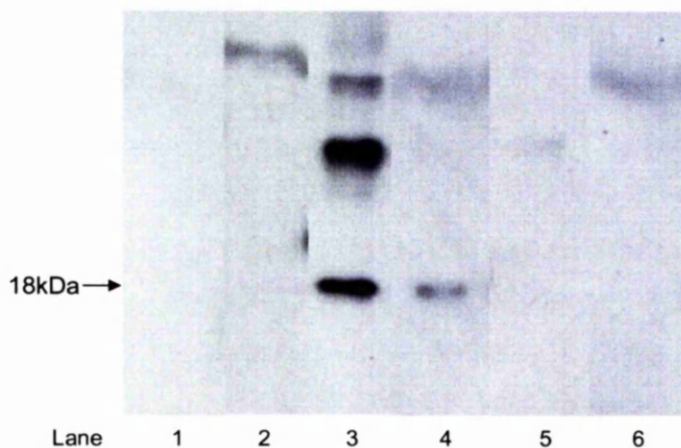
For reference, figure 4.7 shows a similar pattern in the expression of equine ILRAP-IL-18 and PsecI-IL-18. 18 kDa bands are evident in the ILRAP-IL-18 lysate and supernatant, but there is no protein detectable in either PsecI-IL-18 lysate or supernatant suggesting much lower or maybe even non-existent expression of this signal protein.

Figure 4-6 SDS-PAGE gel of feline ILRAP-IL-18 and PsecI-IL-18 protein



Autoradiograph of a 15% SDS-PAGE gel showing ILRAP-IL-18 and PsecI-IL-18 protein from transfections. Lanes are as follows: **lane 1:** pCI-neo cell lysate, **lane 2:** pCI-neo supernatant, **lane 3:** ILRAP-IL-18 cell lysate, **lane 4:** ILRAP-IL-18 supernatant, **lane 5:** PsecI-IL-18 cell lysate, **lane 6:** PsecI-IL-18 supernatant.

Figure 4-7 SDS-PAGE gel of equine ILRAP-IL-18 and PsecI-IL-18 protein



Autoradiograph of a 15% SDS-PAGE gel showing ILRAP-IL-18 and PsecI-IL-18 protein from transfections. Lanes are as follows: **lane 1:** pCI-neo cell lysate, **lane 2:** pCI-neo supernatant, **lane 3:** ILRAP-IL-18 cell lysate, **lane 4:** ILRAP-IL-18 supernatant, **lane 5:** PsecI-IL-18 cell lysate, **lane 6:** PsecI-IL-18 supernatant.

4.3.3 *In vitro* bioactivity of feline IL-18 protein using KG-1 bioassay

The KG-1 bioassay measures the level of human IFN γ production produced by KG-1 cells on exposure to a given dilution of IL-18 protein. It should be noted here that these bioassay data were produced from a series of bioassay experiments used to establish suitable sample dilutions and antibody levels. In producing preliminary data for this final experiment, the bioassay was found to be a robust and repeatable assay for the detection of bioactivity in feline IL-18 protein samples.

4.3.3.1 Negative control samples

Both caspase-digested pCI-neo cell lysate in CB (figure 4.8) and pCI-neo cell lysate and supernatant in PBS (figure 4.9) demonstrated background levels of hIFN γ production. In addition, caspase-digested pCI-neo incubated with feline IL-18 specific antibody (figure 4.8) elicited background levels of IFN γ stimulation.

4.3.3.2 IL-18 transfection samples

Cell lysates of all feline IL-18 transfections apart from PsecI-IL-18 demonstrated the dose dependent production of human IFN γ using this system. Cell lysate dilution ranges were selected for each IL-18 construct according to where background levels of bioactivity were reached. Assays were therefore performed for mature-IL-18, ILRAP-IL-18 and PsecI-IL-18 at a dilution range of 1:1024 to 1:32 (figure 4.9) and graphs for pro-IL-18 at a range of 1:20000 to 1:1250 (figure 4.8).

Caspase-digested pro-IL-18, mature IL-18 and ILRAP-IL-18 cell lysates demonstrated dose dependent production of IFN γ from KG-1 cells whereas non-digested pro-IL-18 stimulated background levels of hIFN γ production. Assuming different IL-18 proteins were comparable in terms of KG-1 IL-18 receptor binding, the fact that similar hIFN γ levels were produced at these dilutions again suggests that pro-IL-18 protein expression is approximately 20-fold greater than ILRAP- and mature-IL-18 expression. Additionally, mature-IL-18 cell lysate demonstrates approximately half the level of hIFN γ production than ILRAP-IL-18 cell lysate at a

given dilution. PsecI-IL-18 protein exhibited almost background levels of IFN γ production at all dilutions.

Figure 4.8 also shows that the hIFN γ elicited by caspase-digested pro-IL-18 cell lysate was suppressed by incubation with a feline IL-18-specific polyclonal antibody. Incubation with a non-specific FeLV rabbit polyclonal antibody produced no suppression of bioactivity. Suppression of this bioactivity by an IL-18-neutralising antibody suggests that IFN γ stimulation is specific to IL-18 protein present in the sample.

As previously stated, pro-IL-18 and mature-IL-18 supernatants demonstrated background levels of bioactivity and were not included in this experiment. However, comparisons of transfection supernatants were performed for ILRAP-IL-18 and PsecI-IL-18. ILRAP-IL-18 supernatant demonstrated dose dependent IFN γ production whereas PsecI-IL-18 supernatant elicited background levels of IFN γ from KG-1 cells (figure 4.9).

Figure 4-8 KG-1 assay of pro-IL-18 with antibodies and controls

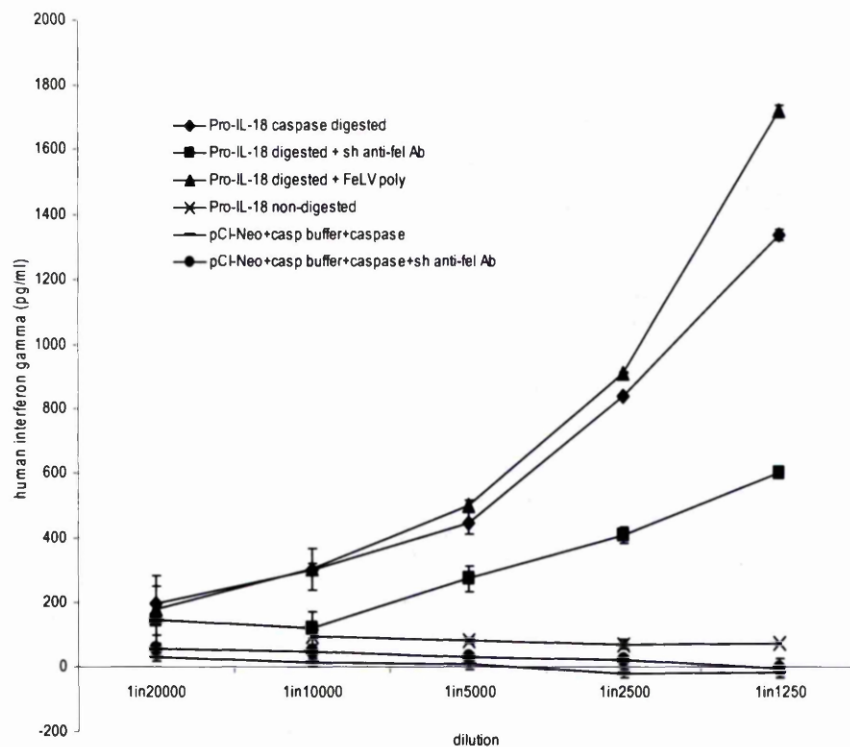
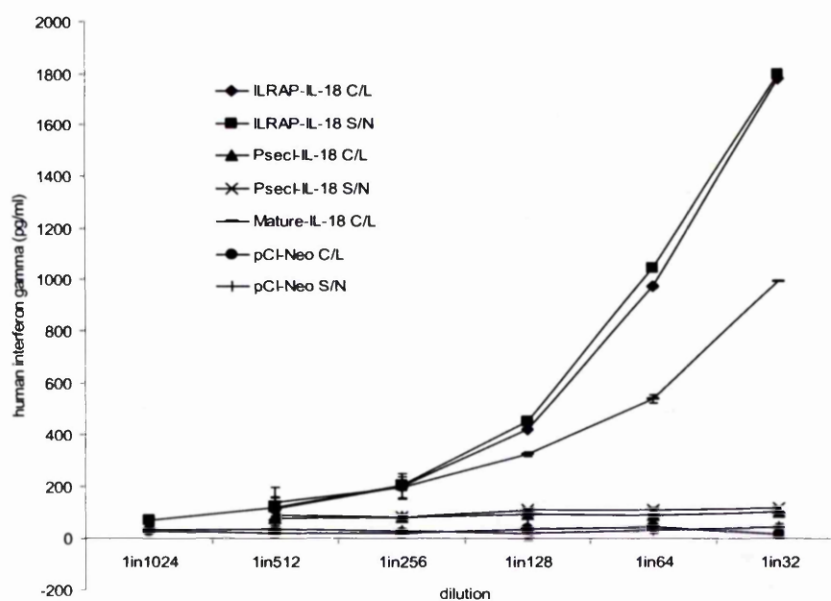


Figure 4-9 KG-1 assay of mature-IL-18, PsecI-IL-18 and ILRAP-IL-18

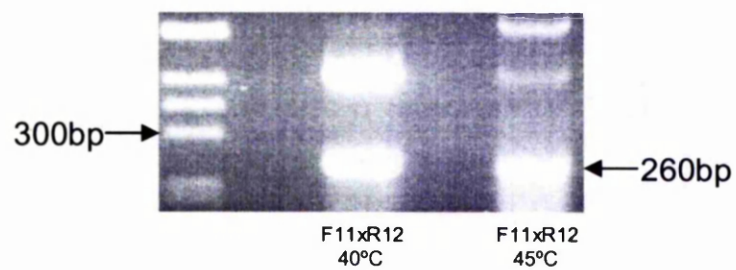
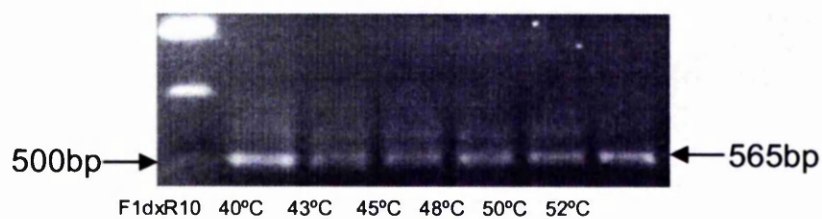
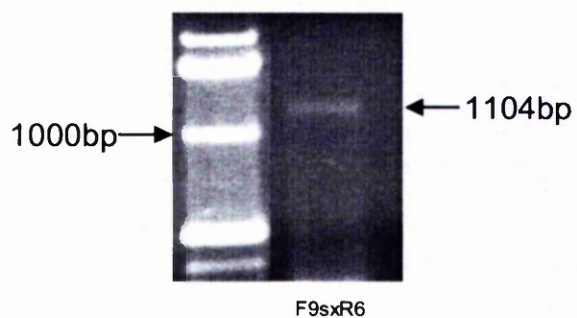
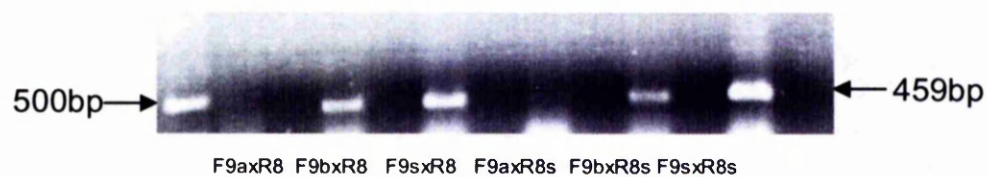


4.3.4 Amplification of the feline IL-18 receptor

The initial set of IL-18 receptor primers yielded several fragments of F9 x R8 and also an F9 x R6 fragment using an annealing temperature of 45°C. Confirmation of positive clones using the ABI Prism system allowed design of subsequent primers based on exact feline sequence. These primers, used in combination with degenerate primers based around the start and stop codon sequences, amplified F11 x R12 and F1d x R10 fragments at annealing temperatures as shown in figure 4.10.

Confirmed feline IL-18R α sequence at the start and stop codon allowed redesign of F1d and R12 primers to produce F3DN and R12NL respectively. Combinations of these and new primers yielded F3DN x R8s, F13 x R12NL and F15 x R12NL, F9SF x R16 and F9SF x R17 fragments. As shown in figure 4.11, this resulted in a series of IL-18R fragments across the 1650 base pair sequence. The final feline IL-18R α sequence was confirmed by producing a consensus of sequences produced from each of these fragments using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin) and the encoding amino acid sequence was elucidated (figure 4.12). The sequence was assigned a GenBank Accession number AY 160954.

Figure 4-10 PCR fragments of feline IL-18 receptor



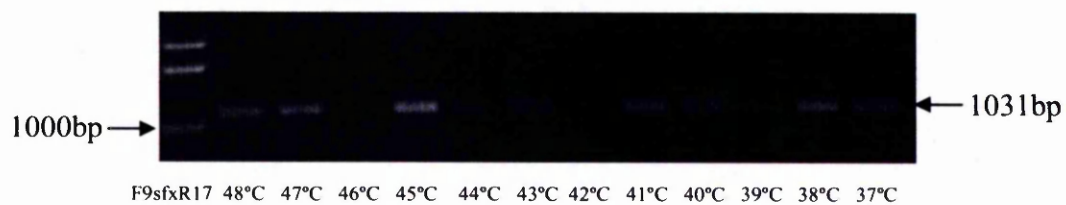
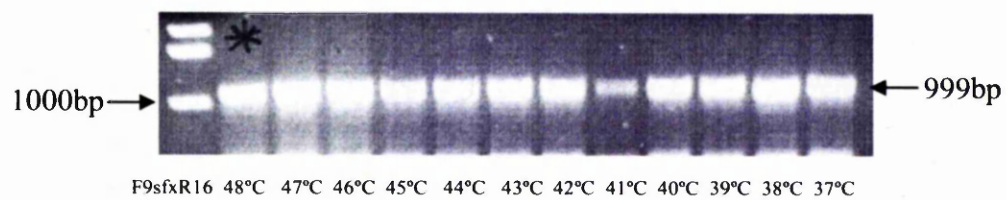
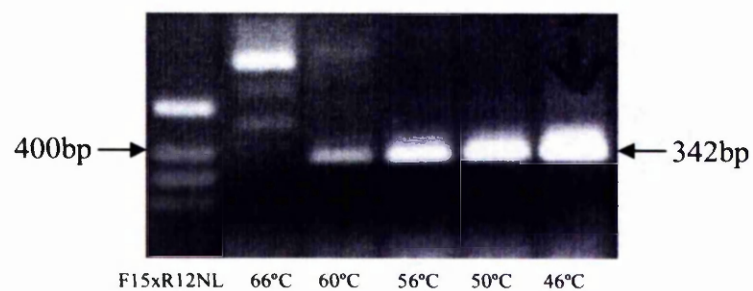
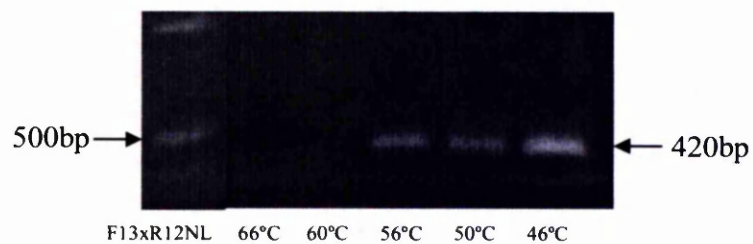
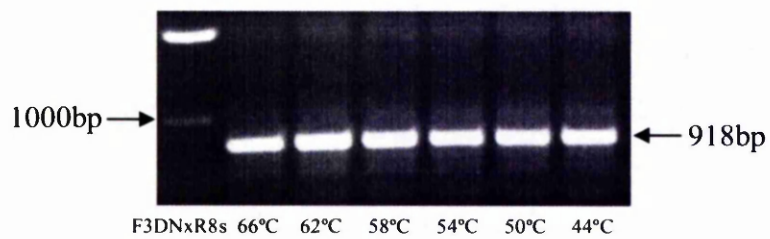


Figure 4-11 Diagram showing feline IL-18 receptor fragments

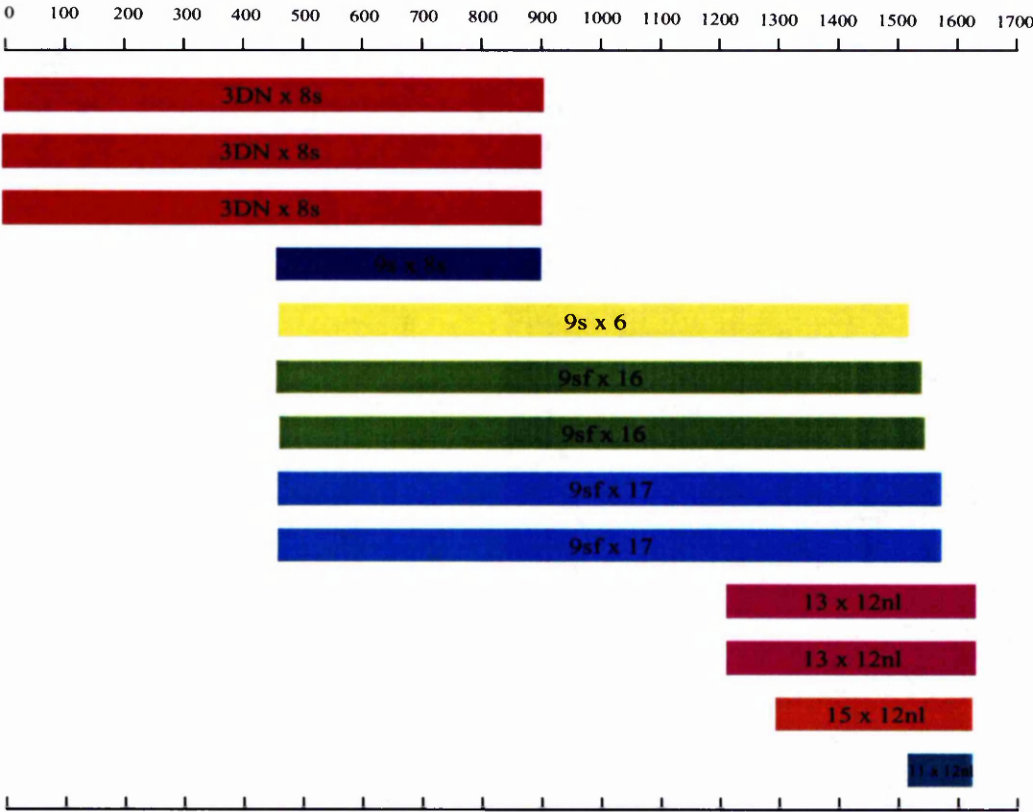


Figure 4-12 Feline IL-18R α nucleotide and amino acid sequence

```

atgcgatcatatagaactgctcttaacgcttttggcacttatggtttacaagcacctcagaa 60
  M R H I E L L L T L L A L M F T S T S E
acatgtattttcacgtcatagcatcactgcagtggaaggggaatttttctatctgagatac 120
  T C I S R H S I T A V E G E F F Y L R Y
tgctcatcagcatctgagcagcatagtgaaaaaacagcataaaatggtacaagagcagt 180
  C S S A S E H D S E K N S I K W Y K S S
gggtcacatggacgcattgagctaaactcaagcagttccccagaattactttgcacgat 240
  G S H G R I E L N S S S S P R I T L H D
tatgttttggagttttggccagttgagttggaggacagtggtatcttactttttccaaatg 300
  Y V L E F W P V E L E D S G S Y F F Q M
ggaaatgatactcgtaaatggaattaaatgtcattggaagaagtaaaagcagctgtttt 360
  G N D T R K W K L N V I G R S K S S C F
gttgaaaaactactaactagtaaaactgtagaagttcagaaatctttgcacgtagcctgt 420
  V E K L L T S K T V E V Q K S L H V A C
aaacatgactactttcaaccattggccaatagaacttcactgtataagaactgtgaaaag 480
  K H D Y F Q P L A N R T S L Y K N C E K
ataaacaatggtacaaacccagttttacagaagaatgcagagtttgaagatcagggatat 540
  I N N G T N P V L Q K N A E F E D Q G Y
tacacctgtgtgttttccatccctcataatggaaaactatttaatgtcaccaaaaccatc 600
  Y T C V F S I P H N G K L F N V T K T I
aacgtaacaatcgttggagatcgcagtaaaataattcctgttcttcttggaccaaagctt 660
  N V T I V G D R S K I I P V L L G P K L
aaccgtgtgaaggtggaattaggaaaagatgtaaacctcaattgctctgctttggcgaat 720
  N R V K V E L G K D V N L N C S A L A N
gaaaaggatcagatttatttgaacttgtgggatgaaatggaaggagcccaatgtacat 780
  E K D Q I Y W N L W D E N G K E P N V H
gaagagaacgtaaaaaaaaaataggactctggatggcaaattgtatgtgtcaagaatgttg 840
  E E N V K K N R T L D G K L Y V S R M L
aaaaatcgagaatattaatgcaaaaaatctaaaattttcatataattgcactgtggccagt 900
  K I E N I N A K N L K F S Y N C T V A S
gaggaggacacagacaccataaaacttcgtcttgttgaaaaagaagatatggctgatatc 960
  E G G T D T I N F V L L K K E D M A D I
ccaggctacatcttcaccagaggaatgattgtggctgttttgatctcagtggtagtgtg 1020
  P G Y I F T R G M I V A V L I S V V V V

```

tgccctagtataatgggtgtcatttatagagttgacttggtctatatttatagacatttc 1080
 C L V I M G V I Y R V D L A L F Y R H F
 acgagaaaagatgaaacattaacagatgggaaaacatacgacgcttttgtgtcttaccta 1140
 T R K D E T L T D G K T Y D A F V S Y L
 aaagaatgtggacccgaaaatggagaggagcacacctttgctgtggagattttgcccaag 1200
 K E C G P E N G E E H T F A V E I L P K
 gtgttgaggagaaacactttgggtataagttatgcataatgaaagggatgtagtgcctgga 1260
 V L E K H F G Y K L C I Y E R D V V P G
 ggagctattgttgatgaaatccactcattgatagagaaaagccgaagactgatcattgtc 1320
 G A I V D E I H S L I E K S R R L I I V
 ctaagtaaaagctacatgtcctaataagtcaggtatgaacttgaaagtggaactccatgaa 1380
 L S K S Y M S N E V R Y E L E S G L H E
 gctctggtagaaaggaaaattaaaatcatcttaattgaatttacacctgtcagtgacttc 1440
 A L V E R K I K I I L I E F T P V S D F
 acattcttcccccaatcactaaagcttttgaaatctcacagagttctgaagtggaacgct 1500
 T F F P Q S L K L L K S H R V L K W N A
 gataaacctctgtcgtataactcaaggttctggaagaatcttctgtacctgatgcctgca 1560
 D K P L S Y N S R F W K N L L Y L M P A
 aaagtggtaagcctcgtggaactgaatctgaagtctttcctgttctttccgagctcttaa 1620
 K V V K P R G T E S E V F P V L S E S *

4.4 DISCUSSION

Since its isolation, the biological significance of IL-18 in stimulation of the immune response has led to its use *in vivo* as an immunotherapeutic against pathogens such as bacteria [Neighbors *et al.* 2001], viruses [Tanaka-Kataoka *et al.* 1998] and fungi [Menacci *et al.* 2000]. In addition, IL-18 has been adopted as a potent immunotherapeutic agent against tumours. In this case IL-18 has been used alone and as an adjuvant to other types of tumour therapy by several different methods of delivery [Cao *et al.* 1999; Osaki *et al.* 1999]. IL-18 has also been utilised as an adjuvant to vaccination, making use of its ability to modulate the immune response towards cell mediated immunity [Billaut-Mulot *et al.* 2001; Hanlon *et al.* 2001].

However, the synthesis and secretion of native IL-18 within the cell poses certain challenges to researchers in this field. As mentioned in 4.1.3, IL-18 is synthesised as a biologically inactive precursor molecule pro-IL-18 [Okamura *et al.* 1995], which is cleaved by ICE or caspase-1 to the active molecule, mature IL-18. Mature-IL-18 then carries the natural signal required for secretion across the cell membrane [Gu *et al.* 1997]. Both mature-IL-18 cDNA [Giavedoni *et al.* 2001] and pro-IL-18 cDNA [Oshikawa *et al.* 1999] constructs have been studied. However there are potential processing problems with each of these constructs. Researchers using pro-IL-18 cDNA depend on the presence of caspase-1 producing transfected cells for endogenous cleavage and secretion of the bioactive mature molecule. Those utilising mature-IL-18 cDNA rely on the release of the molecule from injured cells or those undergoing apoptosis as this molecule lacks a natural signalling peptide. In an attempt to overcome these problems, studies have been carried out using mature-IL-18 cDNA fused to signalling peptides to encourage the secretion of the molecule without the requirement for caspase-1 cleavage [Osaki *et al.* 1999; Hanlon *et al.* 2001; Kim *et al.* 2001].

The purpose of this work was to analyse the *in vitro* expression of feline pro- and mature-IL-18 and to establish whether the use of two synthetic signal sequences ILRAP and PsecI demonstrated intracellular expression of protein and successful

protein secretion. Additionally, a system analysing the *in vitro* bioactivity of these constructs was used to investigate activity and IL-18 secretion of these constructs.

4.4.1 *In vitro* protein expression of feline IL-18 constructs

Western blot analysis of transfections of feline IL-18 plasmids demonstrated *in vitro* protein expression of pro-, mature-, and ILRAP-IL-18. This experiment demonstrated that feline pro-IL-18 was strongly expressed in CHO cells and was successfully cleaved to mature-IL-18 using a recombinant caspase-1 enzyme. The lack of mature-IL-18 in the pro-IL-18 supernatant suggests that CHO cells do not naturally produce high levels of caspase-1, thereby preventing secretion of the bioactive molecule. Mature-IL-18 construct demonstrated expression of protein in the cell lysate without release into the supernatant which confirms that the molecule lacks the signalling sequence required to allow secretion from the cell.

In contrast, ILRAP-IL-18 protein was detectable both in the transfection cell lysate and supernatant, which shows that this signal protein is synthesised and successfully secreted from the cells into which it is transfected. This indicates that when fused with feline mature-IL-18, ILRAP provides a suitable signal sequence for IL-18 protein expression and secretion from transfected cells.

4.4.2 Comparison of ILRAP-IL-18 and PsecI-IL-18 *in vitro* expression

To compare their relative levels of protein expression, parallel transfections of ILRAP-IL-18, PsecI-IL-18 and pCI-neo were performed along with a pCI-neo negative control. Again, the feline ILRAP signal construct displayed strong protein expression in the cell lysate of transfected cells. The expression exhibited by feline PsecI-IL-18 in the cell lysate was minimal, only just within the detection limits of this protein detection system. This is indicative of a lower level of PsecI-IL-18 protein expression relative to ILRAP-IL-18, but further work would be required to definitively confirm the low protein expression of this construct. This relative difference in protein expression between ILRAP and PsecI signal protein was also

seen in the equine IL-18 constructs, where there was no evidence of PsecI-IL-18 protein expression in either of the transfection products.

When the levels of IL-18 signal protein in transfection supernatants were analysed, ILRAP-IL-18 demonstrated similar levels of IL-18 protein expression in the supernatant relative to the cell lysate. However, PsecI-IL-18 showed no evidence of IL-18 in the supernatant even though a low level was present within the cell. If both signal constructs secreted protein to the same level, then the relative proportions of IL-18 in supernatant compared to cell lysate would presumably be similar. This therefore implies that ILRAP signal is more effective at bringing about cell secretion than PsecI signal. However it is difficult to make a fair comparison of the protein expression of each supernatant as expression levels of PsecI-IL-18 were so low.

There are several reasons why IL-18 protein expression of ILRAP-IL-18 transfection products may have appeared higher than those of PsecI-IL-18. PsecI-IL-18 protein or mRNA may have expressed at a similar level but have been unstable, degraded within the cell and therefore undetectable using this system. Another possibility is that the PsecI signal peptide was not cleaved by signal peptidase from the primary translation product which may have altered the structure of the IL-18 molecule, preventing binding of the primary antibody. It is possible that lower PsecI-IL-18 expression may be a reflection of low transfection efficiency in this particular experiment. However, in producing this data at least three separate transfections were performed, all of which demonstrated low protein expression.

It should be noted here that the feline PsecI-IL-18 construct was shown *in vivo* to act in combination with IL-12 as an effective adjuvant for a DNA vaccine to FeLV [Hanlon et al. 2001]. In cats inoculated with DNA vaccine and IL-12 plasmid, three of six cats were viraemic and four were latently infected at 15 weeks post challenge. With the coadministration of PsecI-IL-18 plasmid, no cats were viraemic and only one was latently infected at this time. This study implies that the PsecI-IL-18 expression vector must be expressed and secreted at least to some extent in host cells to exert its effect, although it is possible that *in vivo* this protein was released from injured cells or those undergoing apoptosis rather than being secreted using the

synthetic signal. The actual process of intramuscular injection of this type of vaccine may provide the necessary cell death to encourage the release of protein in this way.

In conclusion, this work shows that feline ILRAP-IL-18 signal protein shows a high level of protein expression relative to PsecI-IL-18 in this *in vitro* expression system. It also confirms that ILRAP-IL-18 protein is effectively secreted out of transfected cells, in contrast to pro-IL-18 and mature-IL-18 constructs.

4.4.3 *In vitro* bioactivity of feline IL-18 constructs

Several negative control transfection samples were used in this experiment including pCI-neo cell lysate and supernatant and caspase-digested pCI-neo cell lysate. The purpose of these samples was to establish whether cell lysates or supernatants of transfected cells contained any components other than IL-18 protein, which directly or indirectly induced detectable levels of IFN γ . In addition, a sample of caspase-digested pCI-neo incubated with feline IL-18 specific antibody was also used to establish whether this antibody preparation influenced the level of IFN γ production by KG-1 cells. In all cases, background levels of IFN γ were elicited, confirming that of IFN γ induction by IL-18 samples was due to IL-18 protein rather than any other codelivered transfection products.

When pro-IL-18 cell lysate was used directly in this assay without prior caspase-1 digestion, background levels of activity were detected, whereas digestion with caspase-1 induced dose dependent production of IFN γ from KG-1 cells. This shows that in order to demonstrate bioactivity, the inactive pro-IL-18 precursor must first be cleaved by caspase-1 to release the bioactive mature molecule. This protease cleavage process was confirmed in Western blot analysis, where undigested pro-IL-18 demonstrated a protein band approximately 24 kDa in size, which on caspase-1 digestion displayed an 18 kDa band.

Previous assays detected no activity in transfection supernatants of pro-IL-18 and mature-IL-18, which were therefore excluded from the present experiment. This confirms the Western analysis that showed that neither protein was secreted from the

transfected cell. The cell lysate of pro-IL-18 induced dose dependent IFN γ production at approximately 20-fold higher dilutions than mature-IL-18, which confirms the previous *in vitro* data that required a 1:20 dilution to demonstrate comparable protein bands on the Western blot. As cleavage of pro-IL-18 produces the same mature polypeptide as expressed in a mature-IL-18 transfection, the difference in IFN γ production must be reflective of the relative expression or processing of pro- and mature-IL-18 in transfected cells. This difference may be because *in vivo* IL-18 protein is stored as the pro-IL-18 precursor, which is subsequently processed to allow secretion of bioactive mature-IL-18 [Gu *et al.* 1997; Nakanishi *et al.* 2001a]. Therefore mature-IL-18 synthesised from the mature-IL-18 transfection may be unstable and only have a short half-life in the cell.

To confirm specificity of this response to IL-18 activity, this sample was pre-incubated with a sheep anti-feline polyclonal antibody at a dilution of 34 ng/ μ l. This suppressed hIFN γ production to approximately half its original level. This antibody must therefore recognise regions of IL-18 responsible for attachment of IL-18 to the receptor on the KG-1 cell. The antibody therefore neutralises IL-18 by obstructing receptor binding, preventing the signalling pathway required for IFN γ production.

It is possible however that incubation with the antibody preparation itself could elicit suppression of IL-18 bioactivity. For example the medium used to elute the antibody, or the antibody itself could have an inhibitory effect on the KG-1 cells. Therefore a non-specific polyclonal antibody prepared in the same way was incubated with digested pro-IL-18 cell lysate. This produced no inhibition of IFN γ production; in fact it produced a mild stimulatory effect on IFN γ production in this system. However, the level of activity demonstrated by the inclusion of the negative antibody was so small that this was not found to be significant. These data therefore suggest that the suppression by the sheep anti-feline antibody was due to the neutralising effect of the antibody rather than any inhibitory effect of the antibody preparation.

A comparison of the bioactivity of cell lysates and supernatants of the two IL-18 signal constructs ILRAP- and PsecI-IL-18 were made. Both lysate and supernatant of PsecI-IL-18 transfection failed to elicit IFN γ stimulation from KG-1 cells. The most

likely explanation for this would be a low level of protein expression, which was reflected in the Western blot analysis of this construct. However, the transfections used to compare the bioactivity of these signal constructs were different to those used for Western blot analysis which prevents a direct comparison of Western blot and KG-1 bioassay data. It was therefore not possible to establish whether the lack of activity of PsecI-IL-18 transfection samples was due to low levels of protein present, or to a lack of production of bioactive protein in the sample.

ILRAP-IL-18 cell lysates and supernatants displayed dose dependent induction of hIFN γ from KG-1 cells. This confirms the Western blot data, suggesting that feline ILRAP-IL-18 protein was expressed and readily secreted from transfected cells. In addition it shows that ILRAP-IL-18 produces a protein that is able to bind its associated receptor and stimulate the signalling pathway required to carry out its biological function on target cells.

In conclusion, this *in vitro* information suggests that signal ILRAP-IL-18 holds advantages over all other feline IL-18 constructs studied in this chapter. Firstly the level of bioactive protein expression within the cell appears to be superior to the other signal construct PsecI-IL-18. Additionally this construct displays activity in transfection supernatant, which was not detected in any other feline IL-18 construct. This synthetic signal overcomes the problems experienced using a mature-IL-18 construct, which lacks a natural signal peptide to allow secretion, and the pro-IL-18 construct, which relies on endogenous caspase-1 for cleavage to produce the bioactive molecule. ILRAP-IL-18 was therefore selected as the most appropriate feline IL-18 construct for use *in vivo* as an adjuvant to a DNA vaccine for FeLV described in chapter 5.

Studies using other synthetic signal sequences for IL-18 have also shown success in terms of bioactivity and cell secretion. The fusion of mature-IL-18 to the prepro sequence of human parathyroid hormone produced a construct whose transfection supernatant stimulated IFN γ production in cocultured splenocytes. This confirmed both cell secretion and bioactivity of this construct, which was then used in a recombinant adenoviral vector to induce tumour regression in a murine fibrosarcoma

model [Osaki *et al.* 1999]. Also, the fusion of mature-IL-18 to the human immunoglobulin kappa leader sequence produced a signal construct which was transfected into COS-7 cells. Secretion of this construct across the cell membrane was confirmed by Western blot analysis of transfection supernatant [Kim *et al.* 2001]. Collectively then, these studies, along with the data produced in this chapter, show that bioactive IL-18 protein can be effectively synthesised and secreted in an *in vitro* expression system by fusion to conventional signal sequences.

4.4.4 Feline IL-18 receptor

Total RNA extracted from MYA-1 cells, a feline T lymphoblast cell line derived from peripheral blood, was used to amplify the feline IL-18R α receptor sequence. The RT-PCR amplification system used a *Taq* DNA polymerase enzyme which is a low fidelity enzyme with a higher error rate than proof reading enzymes, estimated to be 2×10^{-5} [Lundberg *et al.* 1991]. As stated in Chapter 3, this rate of error may increase if cycling conditions are not optimal for the enzyme. It is therefore recommended that between three and six amplified fragments are required to establish a consensus sequence [Ennis *et al.* 1990]. In this case, three separate PCR fragments were amplified for each area of the gene to produce a consensus nucleotide and amino acid sequence of the receptor.

The feline IL-18R α receptor was 1620bp in length and contained an open reading frame of 540 amino acids (figure 4.12) (GenBank Accession number: AY160954). Comparison of the feline IL-18R α sequence with published sequences shows 83%, 81% and 70% identity with human, porcine and murine sequence at the nucleotide level, and 72%, 76% and 61% at the amino acid level respectively

The nucleotide sequence of human IL-18R α was found to be identical to human IL-1 receptor-related protein (IL-1Rrp) cDNA, a member of the IL-1 receptor (IL-1R) family [Torigoe *et al.* 1997]. IL-1Rrp, like all members of its family is predicted to comprise a signal peptide, an extracellular region which consists of three immunoglobulin like domains, a transmembrane region and a cytoplasmic region [Parnet *et al.* 1995; Vigers *et al.* 1997]. The amino acid sequence of feline IL-18R α

was compared with IL-18R α of other species using the Pileup program of GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin) as shown in figure 4.13. The areas of homology are shown by the consensus sequence in bold type. The predicted signal peptide, transmembrane region, extracellular and intracellular domains were extrapolated from human and murine predictions [Parnet *et al.* 1995]. The asterisks indicate the positions of conserved cysteine residues which are thought to form the typical intradomain disulphide bonds of the three Ig domains of the extracellular region [Parnet *et al.* 1995; Vigers *et al.* 1997]. Using the GCG package Peptidestructure and Plotstructure programs, the areas of hydrophilicity and hydrophobicity of feline IL-18R α were established. Hydrophobic areas were found between amino acids 1 and 20 and amino acids 325 and 355 (data not shown). These areas corresponded to the predicted signal peptide and transmembrane domains respectively.

The purpose of amplification of the IL-18R α receptor was to investigate its ability to increase the sensitivity of the KG-1 bioassay system. Human KG-1 cells were shown to respond to human IL-18 by the dose dependent production of human IFN γ [Konishi *et al.* 1997]. This assay also detected murine IL-18, but at sensitivity of 100-fold less than human protein. It was found that transfection of KG-1 cells with cDNA encoding murine IL-18R α , produced a stable cell line which when used in the bioassay, significantly increased the sensitivity of the system to murine protein [Taniguchi *et al.* 1998].

It was therefore postulated that if a stable cell line of feline IL-18R α -transfected KG-1 cells was used in this assay, they may demonstrate an increased sensitivity towards feline IL-18 protein. This work could therefore be extended in the future by cloning the full length feline IL-18R α sequence into a single expression vector. This would require either amplification of full sequence using specific primers, or the use of restriction enzyme sites in the sequence to allow ligation of fragments. This construct could then be used to produce a stable KG-1 cell line expressing the feline IL-18R α receptor in a similar way to the murine IL-18R α cell line produced by Taniguchi *et al.* [1998]. Parallel bioassays using untransfected KG-1 cells and the feline receptor

transfected cell line would establish whether KG-1 cells expressing feline IL-18R α were more sensitive to feline IL-18 protein than untransfected cells.

Figure 4-13 Consensus of IL-18R α sequences and predicted domains

Red text -signal peptide
Blue text -extracellular regions
Green text -transmembrane region
Purple text -cytoplasmic domain
Asterisks* -cysteines predicted to form intradomain disulphide bonds of Ig domains of the extracellular region

	1				*	50
fil18r	MRHIELLLTL	LALMFTSTS	ETCISRHSIT	AVEGEFFYL	YCS.SASEHD	
pil18r	MHCDELPLTL	LILMFISTSE	EICISRHHIT	AVEGEPFYLK	CCP.SSSEHK	
hil18r	MNCRELPLTL	WVLISVSTA	ESCTSRPHIT	VVEGEPFYLK	HCS.CSLAHE	
mil18r	MHHEELILTL	CIL.I.VKSAS	KSCIHRSQIH	VVEGEPFYLK	PCGISAPVHR	
Con	M---EL-LTL	--L-----	--C--R--I-	-VEGE-FYL-	-C-----H-	
	51					
fil18r	SEKNSIKWYK	SSGSHGRIEL	NSSSSPRITL	HDYVLEFWPV	ELEDGSGSYFF	
pil18r	NKTTTIKWKY	NE.SHGPTTEL	RSGGSPRIIL	HDYVLEFWPV	EMDDSGSYSC	
hil18r	IETTTKSWYK	SSGSQEHVEL	NPRSSSRIAL	HDCVLEFWPV	ELNDTGSYFF	
mil18r	NETATMRWFK	GSASHEYREL	NNRSSPRVTF	HDHTLEFWPV	EMEDGTYIIS	
Con	-----W-K	---S---EL	---S-R---	HD--LEFWPV	E--D-G-Y--	
	101				*	150
fil18r	QMGNDTRKWK	LNVIGRSKSS	CFVEKLLTSK	TVEVQKSLHV	ACKHDYFQPL	
pil18r	RMGNDTQAWK	LNVIIRRSKNS	WFTEKQVISK	VVEVKKTLQV	PCENNYFQNL	
hil18r	QMKNYTQKWK	LNVIIRNKHS	CFTERQVTSK	IVEVKKFFQI	TCENSYYQTL	
mil18r	QVGNDRRNWT	LNVTKRNKHS	CFSDKLVTSR	DVEVNKSLHI	TCKNPNYEEL	
Con	---N---W-	LNV--R-K-S	-F-----S-	-VEV-K----	-C-----L	
	151				*	200
fil18r	ANRTSLYKNC	EK..INNGTN	PVLQKNAEFE	DQGYTTCVFS	IPHNGKLFNV	
pil18r	ANRTSLYKDC	EK..IDFNFN	LNKKIAEFK	DGGYTCVFF	LHHAGKLFNV	
hil18r	VNSTSLYKNC	KKLLLENNKN	PTIKKNAEFE	DQGYSCVHF	LHHNGKLFNI	
mil18r	IQDTWLKNC	KEI...SKT	PRILKDAEFG	DEGYSCVFS	VHHNGTRYNI	
Con	---T-LYK-C	-----	---K-AEF-	D-GYY-CV--	--H-G---N-	
	201				*	250
fil18r	TKTINVTIVG	DRSKIIPVLL	GPKLNRVKVE	LGKDVNLNCS	ALANEKDQIY	
pil18r	TKTFNITIIG	DHSSIIPALL	GPKLTQVKVE	LGKDTQLNCS	ALLNEKDVVY	
hil18r	TKTFNITIVE	DRSNIVPVLL	GPKLNHVAVE	LGKNVRLNCS	ALLNEEDVIY	
mil18r	TKTVNITVIE	GRSKVTPAIL	GPKCEKVVE	LGKDVELNCS	ASLNKDDLFI	
Con	TKT-N-T---	--S---P--L	GPK---V-VE	LGK---LNCS	A--N--D--Y	
	251					
fil18r	WNLWDENGKE	PNVHEE.NVK	KNRTLKGKLY	VSRMLKIENI	NAKNLKFSSYN	
pil18r	WNTWRENGSD	PNVHEE.EGT	RIRTPDGKWL	ASKTLRIENV	NEKNLNFLYN	
hil18r	WMFGEENGSD	PNIHHEE.KEM	RIMTPEGKWH	ASKVLRIENI	GESNLNVLYN	
mil18r	WSIRKEDSSD	PNVQEDRKET	TTWISEGKLH	ASKILRFQKI	TENYLNVLNLYN	
Con	W----E----	PN--E-----	-----GK--	-S--L-----	----L---YN	
	*					
fil18r	CTVASEGGTD	TINFVLLKKE	DMADIPGYIF	TRGMIVAVLI	SVVVVCLVIM	
pil18r	CTVASKGGTD	TKSFILLRKE	DMADIPGHVF	TRGMIVAALI	SVSIVCLVIM	
hil18r	CTVASTGGTD	TKSFILVRKA	DMADIPGHVF	TRGMIIAVLI	LVAVVCLVTV	
mil18r	CTVANEEAID	TKSFVLVRKE	.IPDIPGHVF	TGGVTVLVLA	SVAAVCIVIL	
Con	CTVA-----D	T--F-L--K-	---DIPG--F	T-G-----L-	-V--VC-V--	
	351					
fil18r	GVIYRVDLAL	FYRHFTRKDE	TLTDGKTYDA	FVSYLKECGP	ENGEETFAV	
pil18r	GVIYRVDLAL	FYRHFTGRDE	TLTDGKTYDA	FVSYLKECRP	ENGEEYTFV	
hil18r	CVIYRVDLVL	FYRHLTRRDE	TLTDGKTYDA	FVSYLKECRP	ENGEETFAV	
mil18r	CVIYKVDLVL	FYRRIAERDE	TLTDGKTYDA	FVSYLKECHP	ENKEEYTFV	
Con	-VIY-VDL-L	FYR-----DE	TLTDGKTYDA	FVSYLKEC-P	EN-EE-TFAV	

401 450
 fill18r EILPKVLEKH FGYKLCIYER DVVPGGAIVD EIHSLIEKSR RLIIVLSKSY
 pill18r EILPRVLEKH FGYKLCIFER DVVPGRAVVE EIHSLIEKSR RLIIVLSKSY
 hill18r EILPRVLEKH FGYKLCIFER DVVPGGAVVD EIHSLIEKSR RLIIVLSKSY
 mill18r ETLPRVLEKQ FGYKLCIFER DVVPGGAVVE EIHSLIEKSR RLIIVLSQSY
 Con E-LP-VLEK- FGYKLCI-ER DVVPG-A-V- EIHSLIEKSR RLIIVLS-SY

451 500
 fill18r MSNEVRYELE SGLHEALVER KIKIILIEFT PVSDFTFFPQ SLKLLKSHRV
 pill18r MSNEVRYELE SGLHEALVER KIKIILIEFT PVGDFTFLPQ SLKLLKSHRV
 hill18r MSNEVRYELE SGLHEALVER KIKIILIEFT PVTDFTFPLPQ SLKLLKSHRV
 mill18r LTNGARRELE SGLHEALVER KIKIILIEFT PASNITFLPP SLKLLKSYRV
 Con --N--R-ELE SGLHEALVER KIKIILIEFT P----TF-P- SLKLLKS-RV

501 545
 fill18r LKWNADKPLS YNSRFWKNLL YLMPAKVVKP RGTESEVFPV LSES*
 pill18r LKWKAEKSL S YNSRFWKNLR YLMPAKTVKP CGDESEVLPV LSQA~
 hill18r LKWKADKSL S YNSRFWKNLL YLMPAKTVKP GRDEPEVLPV LSES*
 mill18r LKWRADSP.S MNSRFWKNLV YLMPAKAVKP WREESEARSV LSAP*
 Con LKW-A----S -NSRFWKNL- YLMPAK-VKP ---E-E---V LS---

4.5 CONCLUSION

The purpose of this work was to design a signal-IL-18 construct and analyse its expression and bioactivity in comparison to pre-existing constructs cloned in our laboratories. We cloned mature-IL-18 fused to a signal sequence of the human IL-1 β gene called IL-1 β receptor antagonist protein (ILRAP). This construct was found to be expressed within transfected cells and to be readily secreted across the cell membrane. Protein secretion could not be detected in any other feline IL-18 constructs. In addition, our group applied a KG-1 bioassay previously used to detect human IL-18 in the detection of feline IL-18 protein [Konishi *et al.* 1997]. This assay confirmed *in vitro* bioactivity of feline IL-18 constructs and demonstrated bioactivity of ILRAP-IL-18 transfection supernatant. Finally, the feline IL-18R α receptor sequence was established which may be used in future work to increase the IL-18 bioassay sensitivity to feline IL-18 protein.

CHAPTER 5: DNA VACCINATION TRIAL

5 DNA VACCINATION TRIAL

5.1 INTRODUCTION

5.1.1 Types of viral vaccine

Several categories of FeLV vaccines are either commercially available or have been studied experimentally *in vivo*. These include attenuated virus, inactivated whole virus, subunit vaccines, live vector vaccines and DNA vaccines. All of these preparations have their own advantages and disadvantages. Live vaccines, similar to a pathogen *in vivo*, are able to stimulate both cellular and humoral immunity, providing strong protection. However, these vaccines have the potential to revert to virulence, causing infection in some animals and may be pathogenic in pregnant or immunosuppressed individuals. Inactivated whole virus vaccines are considered safer but also have the potential to cause disease if the inactivation process is not complete. Inactivated whole virus vaccines expose the host to all antigens present on the whole pathogen but do not tend to stimulate CD8⁺ CTLs from MHC I association as they are not synthesised as endogenous antigen in the APC. They are also expensive to produce and require adjuvants.

Viral subunit vaccines may be extracted from native virus or may be recombinant preparations. These vaccines are generally classed as safe, as the host is only exposed to the antigenic part of the pathogen. However, the inclusion of only certain antigens limit the immune response compared to live and inactivated whole virus vaccines, which expose the host to all antigenic proteins. As described above, these subunits are also unable to stimulate CTLs unless administered with adjuvants.

Live vector vaccines consist of attenuated strains of bacteria or viruses, which encode specific antigenic proteins of a pathogen. Once inoculated, these vectors undergo their normal life cycle and are able to stimulate both CD8⁺ and CD4⁺ immune cells. The vector carrier itself also acts as an inherent adjuvant to the vaccine due to its high immunogenicity. However, questions have arisen over the repeated use of these vectors in the host and as in subunit vaccines only certain antigens are delivered,

which limits the capacity of the immune response. Again, reversion to pathogenicity is a potential risk using these vectors and vaccination could lead to infection in immunocompromised individuals.

The potential advantages of DNA immunisation have been explained in detail in the main introduction. These advantages include induction of both cellular and humoral immunity and no reversion to virulence due to inclusion of only partial genomic sequence. In addition, this type of vaccine is heat stable and easy and inexpensive to prepare and purify. As mentioned previously, however, the use of selected antigens limits the immune response compared to exposure to the whole pathogen.

5.1.2 Efficacy of FeLV vaccination

In a FeLV vaccination study, there are two basic parameters by which the efficacy of a vaccine is assessed. Firstly, the level of resistance of vaccinates to live viral challenge must be established. The second parameter that must be assessed is the proportion of persistently infected animals in the vaccinated group compared to the control animals.

Experimental parameters vary widely between studies such as strain of virus, age of cats, time of challenge, definition of persistent viraemia and method of FeLV challenge used. This experimental variation between FeLV studies is summarised in table 5.1. It is clear that this variation presents difficulties when comparing the efficacy of vaccines. Table 5.2 shows the proportions of persistently infected animals in vaccine groups and control groups for various studies. The individual parameters of each experimental study must be taken into account in the analysis of the data produced.

Tables 5.1 and 5.2 were derived from a review of FeLV vaccination by Sparkes [1997]. The various vaccines currently commercially available in the UK and USA are summarised in table 5.3.

Table 5-1 Viral challenge parameters used in FeLV vaccination studies

Study	Age at challenge	Virus	Dose of virus (f.f.u.)	Method of challenge	Definition of PV	% PV in controls
Haffer <i>et al.</i> 1990	17-25W	FeLV-A/Rickard	10 ⁶ on day 1, 10 ^{5.8} on day 2	O/N + S	Antigenaemia at 12W remaining for 6W	6/10 (60%)
Clark <i>et al.</i> 1991	16-19W	FeLV-A/Glasgow	2 x 10 ⁵	I/P	viraemia 12W onwards	14/20 (70%)
Hines <i>et al.</i> 1991	14-19W	FeLV-A/Rickard	Not specified	O/N + S	Antigenaemia at 12W	39/45 (87%)
Legendre <i>et al.</i> 1991	16W	FeLV-A/1161, FeLV-A/CT600 and 2 FeLV-A field isolates	~10 ³ on day 1 and again at day 7 field isolates: 0.8ml blood day 1 + day 8	I/P + S or blood I/V	Viraemia 31W and for 8W prior to this	7/11 (64%)
Lehmann <i>et al.</i> 1991	14-15M	FeLV-A/Glasgow	10 ⁶	I/P	Consistently positive up to and including 24W	10/12 (83%)
Pedersen and Johnson, 1991	15-28W	FeLV-A/CT600, FeLV-A/Rickard	Not specified	O/N + S	Antigenaemia for more than 4W and at 16W	11/12 (92%)
Pollock <i>et al.</i> 1991	Not specified	FeLV-A/Rickard	10 ^{5.7}	O/N + S	Antigenaemia after 8W	50/81 (62%)
Pollock <i>et al.</i> 1991	Not specified	FeLV-A/NCE	10 ^{5.7}	S/C	Antigenaemia after 8W	3/5 (60%)
Sebring <i>et al.</i> 1991	Not specified	Not specified	Not specified	I/P + S	Not specified	4/4 (100%)
York <i>et al.</i> 1991	16-18W	FeLV-A/Richard	Not specified	I/N + I/M + S	Antigenaemia of more than 4W	14/22 (64%)
Pedersen, 1993	9-10M	FeLV-A/CT600	4 x 10 ⁴ T.C. infectious doses at day 1, 3, 5 and 8	O/N + S	Antigenaemia at 12W	10/10 (100%)
Lafrado, 1994	13W	FeLV-A/Richard	Natural exposure	Natural exposure	Antigenaemia at 12W onwards	1/26 (4%)
Jarrett <i>et al.</i> 1996	14W	FeLV-A/Glasgow-1	1 x 10 ⁵	I/P	Antigenaemia at 12W and for at least 6W	7/8 (88%)
Jarrett <i>et al.</i> 1996	14W	FeLV-A/Glasgow-1, FeLV-B/Sarma and FeLV-C/Sarma	2.2 x 10 ⁶ , 2.2 x 10 ⁶ and 6.5 x 10 ⁵ respectively	O/N	Antigenaemia at 12W and for at least 6W	5/6 (83%)
Gueguen <i>et al.</i> 2000	14-15W	FeLV-A/Glasgow-1	1.18 x 10 ⁶	O/N	Antigenaemic on 5 or more occasions or three consecutive tests	8/10 (80%)
Hanlon <i>et al.</i> 2001	20-22W	FeLV-A/Glasgow-1	2 x 10 ⁵	I/P	Viraemia at 15W	3/6 (50%)
Harbour <i>et al.</i> 2002	15-17W	FeLV-A/Glasgow-1	1 x 10 ⁶ on day 0, 2, 4 and 8	O/N	Viraemia or antigenaemia for 3 consecutive weeks, or 5 occasions between 3-15W post-challenge	10/12 (83%)

W = weeks, M = months, O/N = oronasal, I/N = intranasal, I/P = intraperitoneal, S/C = subcutaneous, I/M = intramuscular, S = corticosteroid administration

Table 5-2 Rate of FeLV infection in the vaccinates and controls of previous studies

Vaccine	Study	VACCINATED ANIMALS			CONTROL ANIMALS		
		Number	Combination of persistent and transient viraemia	Latent infection	Number	Combination of persistent and transient viraemia	Latent infection
Leucocell 2	Haffer <i>et al.</i> 1990	25	16 (64%)	NT	10	10 (100%)	NT
	Legendre <i>et al.</i> 1991	12	8 (67%)	11 (92%)	11	10 (91%)	9 (82%)
	Pollock <i>et al.</i> 1991	148	23 (16%) pers only	NT	81	50 (62%)	NT
	Pollock <i>et al.</i> 1991	14	1 (7%) pers only	NT	5	3 (60%)	NT
	Sebring <i>et al.</i> 1991	4	2 (50%) pers only	NT	4	4 (100%)	NT
	Tizzard <i>et al.</i> 1991	10	4 (40%) pers only		18	13 (72%)	
	Lafrado 1994	26	0 (0%)	NT	26	5 (19%)	NT
	Jarrett <i>et al.</i> 1996	12	10 (83%)	NT	8	8 (100%)	NT
Leucat/VacSYN	Legendre <i>et al.</i> 1991	12	8 (67%)	10 (83%)	11	10 (91%)	9 (82%)
	Pedersen and Johnson, 1991	18	10 (56%) pers only	NT	12	11 (92%)	NT
	Sebring <i>et al.</i> 1991	4	3 (75%)	NT	4	4 (100%)	NT
	Jarrett <i>et al.</i> 1996	12	12 (100%)	NT	8	8 (100%)	NT
	York <i>et al.</i> 1991	43	2 (5%)	NT	22	14 (64%)	NT
Fel-O-Vax	Legendre <i>et al.</i> 1991	12	0 (0%)	5 (42%)	11	10 (91%)	9 (82%)
	Sebring <i>et al.</i> 1991	4	0 (0%) pers only	NT	4	4 (100%)	NT
	Sebring <i>et al.</i> 1991	90	4 (4%) pers only		58	53 (91%)	
Fevaxyn	Hines <i>et al.</i> 1991	144	22 (15%)	10/96 transiently or aviraemic animals	45	45 (100%)	6/8 transiently viraemic controls
	Pedersen 1993	10	1 (10%)	0 (0%)	10	10 (100%)	10 (100%)
Leucogen	Clark <i>et al.</i> 1991	20	12 (60%)	3 (15%)	20	20 (100%)	NT
	Lehmann <i>et al.</i> 1991	18	3 (17%)	1 (6%)	12	12 (100%)	10 (83%)
	Jarrett <i>et al.</i> 1996	12	5 (42%)	NT	8	8 (100%)	NT
	Jarrett <i>et al.</i> 1996	6	1 (17%)	NT	6	5 (83%)	NT

5.1.3 Initial development of FeLV vaccine formulations

After the isolation of FeLV, studies into transmission and immunity suggested that the development of a vaccine to the virus was feasible. Firstly it was shown that FeLV is transmitted mainly horizontally via contact with infected cats, rather than by genetic transmission [Hardy *et al.* 1973a]. Those cats that became immune to the virus were also shown to develop virus-neutralising antibodies (VNAb) [Jarrett *et al.* 1973]. In addition, kittens were shown to obtain maternally derived VNABs from the milk, which conferred resistance to the virus [Jarrett *et al.* 1977].

The first vaccine strategy to be attempted involved the inoculation of FeLV-infected FEA cells into uninfected cats. When large amounts of cells were administered, high antibody titres were elicited but these cats also demonstrated virus in the bone marrow at euthanasia. Lower levels of FEA-infected cells produced a significant antibody response with no viral infection at necropsy [Jarrett *et al.* 1973]. Subsequent to this study, a feline lymphoblastoid cell line (FL74) infected with FeLV produced by Theilen *et al.* [1969], was inoculated into cats and found to elicit high anti-FOCMA antibodies and resistance to subsequent viral challenge. In addition, the virus produced from this cell line was shown to have very low infectivity and cats involved in the experiment were not infected at euthanasia [Jarrett *et al.* 1975].

The obvious problem with this vaccine strategy was the potential for the vaccine to infect the host with virus. The safer alternative was therefore the development of inactivated vaccines. A study was performed comparing the efficacy of live virus cultured from FL74 cells, formaldehyde-inactivated whole virus and formaldehyde-inactivated whole FL74 cells. Both inactivated vaccines were found to be safe but elicited no VNABs, anti-FOCMA or resistance to infection. The live viral vaccine in this study was found to be safe, produce strong antibody responses and stimulate resistance to viral challenge [Pedersen *et al.* 1979]. However, the presence of live virus in this formulation still had the potential risk of infection and disease in vaccinates even though it was found to be of low infectivity [Jarrett *et al.* 1975]. When inactivated FeLV and inactivated whole FL74 cells were used as a combined vaccine, the anti-FOCMA antibody response was lower than if inactivated whole cells

were used alone [Olsen *et al.* 1977]. This suggested that FeLV components may have been inducing immunosuppression. One study showed that a vaccine of killed FL74 cells combined with purified p15E polypeptide was associated with an increased risk of developing progressive fatal fibrosarcoma following challenge with feline sarcoma virus (FeSV), which implicated this structural protein in immunosuppression [Mathes *et al.* 1979]. However, the suggestion that proteins associated with whole killed virus were immunosuppressive has been disputed by other studies of inactivated virus vaccines. For example, cats inoculated with inactivated whole FeLV vaccine followed by challenge produced smaller tumours, a lower rate of persistent viraemia and increased protection compared to control animals [Pedersen *et al.* 1986]. As a result it has become generally accepted that FeLV p15E does not generate immunosuppression and may enhance efficacy of vaccines [Hoover *et al.* 1991]. This preliminary work into inactivated virus vaccines led to the first commercial FeLV vaccine.

5.1.4 The first commercial vaccine for FeLV

The first commercial vaccine was developed by refining the method used by Olsen *et al.* [1977], to produce FeLV antigen from FL74 cells. Antigen was recovered from large volumes of cell culture [Wolff *et al.* 1979] by concentrating tissue culture fluids containing FeLV glycoproteins and tumour antigens in a serum-free medium. The vaccine was emulsified in Freund's adjuvant and inactivated by exposure to UV light. Eighty one percent of vaccinated cats were protected against viraemia and high anti-viral and anti-tumour antibodies were elicited [Lewis *et al.* 1981]. The vaccine contained protein from all three FeLV subgroups and a suitable vaccination regime of three doses was initially found to be efficacious against viraemia and incidence of tumour [Sharpee *et al.* 1986]. This vaccine, Leukocell (Norden Laboratories, Nebraska), became commercially available in 1985.

Subsequently, further work enhanced the antigenic content and therefore immunogenicity of the preparation and produced the second-generation vaccine Leukocell-2. Studies demonstrated that a two-dose programme, three weeks apart provided an effective immunoprophylactic regimen [Haffer *et al.* 1990]. A degree of

controversy arose from this work regarding the efficacy of this preparation. Several studies provided evidence in favour of the effectiveness of the vaccine. One group showed that Leukocell protected three times as many cats against viraemia than placebo-inoculated cats after natural challenge [Pollock and Scarlett 1990], and another showed Leukocell-2 protected 100% of cats against viraemia [Lafrado *et al.* 1994]. In the latter however, only 19% control cats (5 of 26) tested positive for FeLV and 15% of these were transiently infected. In this case the preventable fraction (PF) was found to be 100%, as calculated by the following equation [Loar, 1993]:

$$\text{PF (\%)} = \frac{\% \text{ control cats with persistent viraemia (PV)} - \% \text{ vaccinates with PV}}{\% \text{ controls with PV}} \times 100$$

This value gives a measure of the protective effect of a vaccine in excess of natural immunity. In the study performed by Lafrado *et al.* [1994], although PF is 100% only 1 of the 26 control animals were persistently viraemic. This study was therefore not an effective test of the efficacy of the vaccine, despite an impressive PF valuation.

Other studies have strongly questioned the proficiency of this vaccine. One group showed Leukocell to only be 17% effective against viraemia [Pedersen and Johnson, 1991] and another study using natural challenge, demonstrated 70% viraemia in Leukocell vaccinates compared to 64% unvaccinated animals [Legendre *et al.* 1990].

More recently a study was performed to ascertain the duration of immunity induced by Leukocell 2 vaccination. Animals were vaccinated at 9 and 12 weeks of age and were challenged a year later with oronasal inoculation of virus. Fourteen out of eighteen cats (80%) were protected from viraemia compared to 60% (nine of fifteen) control animals. It was concluded from this study that a significant proportion of cats were protected using this vaccine a year after immunisation [Harbour *et al.* 2002].

5.1.5 Whole inactivated viral vaccines

5.1.5.1 Leucat/VacSYN (Rhone-Merieux/Synbiotics)

Several vaccines comprising killed whole virus were developed and commercialised between 1989 and 1991. One vaccine, subsequently marketed as Leucat or VacSYN (Rhone-Merieux/Synbiotics), consisted of inactivation of FL74 culture fluid by ethylenimine compound. In a study of this vaccine, 2 of 43 cats (5%) developed persistent viraemia after challenge compared to 14 of 22 (64%) control animals [York and York 1991]. Another trial compared the efficacy of Leucocell-2, Leucat and Leucogen, a recombinant subunit vaccine. Of twelve cats in each vaccine group, 10 and 12 animals became persistently viraemic after challenge in the Leukocell-2 and Leucat groups respectively [Jarrett and Ganière 1996].

5.1.5.2 Fel-O-Vax (Fort Dodge)

In another study, a molecularly cloned FeLV-A virus was inactivated and an adjuvant included. This vaccine was used in an experiment alongside the original Leukocell commercial vaccine. The whole killed vaccine demonstrated 100% protection from viraemia whereas all control animals became persistently viraemic. The Leukocell vaccine only showed 43% protection from challenge [Sebring *et al.* 1991]. This whole killed vaccine was subsequently marketed in 1989 as Fel-O-Vax (Fort Dodge).

A natural challenge experiment comparing the efficacy of Leucocell-2, Fel-O-Vax and VacSYN was performed. Of these preparations Fel-O-Vax showed complete protection from viraemia and was the only vaccine to demonstrate statistically significant protection from challenge. In addition, after 31 weeks of exposure to natural challenge, no cats were found to contain latent infection [Legendre *et al.* 1991].

5.1.5.3 Fevaxyn FeLV (Solvay-Dulphar/Fort Dodge)

Similarly, Fevaxyn FeLV (Solvay-Dulphar/Fort Dodge), which came on the market in 1991, comprises cell culture fluids from FeLV-A and -B infected feline cells. The

virus produced was inactivated, concentrated and adjuvanted. A study showed that this vaccine protected 92% vaccinates from viraemia where 87% of the control group were persistently viraemic. When latency was investigated, 10% of vaccinates harboured FeLV infection compared to 75% of control cats [Hines *et al.* 1991].

A subsequent study found that Fevaxyn FeLV vaccine produced complete protection against viraemia and latency where all control cats became viraemic. Eight of the ten cats in the trial demonstrated virus neutralising antibodies to the virus at 5 weeks post-challenge [Pedersen, 1993].

Of the three inactivated whole virus vaccines described, only Fevaxyn FeLV is currently available in UK.

5.1.6 Genetically engineered vaccines: recombinant gp70 subunit vaccine (Leucogen)

Prior to the early 1990s, several groups investigated the use of a vaccine consisting of envelope glycoproteins of FeLV, as antibodies to these proteins have been associated with protection from infection [Russell and Jarrett, 1978a; Lutz *et al.* 1980].

Although the use of glycoproteins recovered from infected cell culture was associated with low immune protection [Salerno *et al.* 1978; Pedersen *et al.* 1986], the development of a pure recombinant gp70 subunit vaccine was pursued due to potential advantages over existing commercial vaccines at that time. Firstly the use of a subunit vaccine excludes the infection potential that killed vaccines hold if there is incomplete inactivation of virus. Vaccines such as Leukocell also have the theoretical potential to cause sensitisation and allergy as it is likely that they contain extraneous proteins. However, a recombinant vaccine of pure protective antigen eliminates this possibility.

Recombinant FeLV-A gp70 antigen was expressed in *E. coli* and purified by gel filtration chromatography and anion exchange chromatography. Aluminium hydroxide was used as a carrier of the immunogen, which was combined with the

adjuvant QS-21, purified from Saponin. A study of the immune response to this vaccine in cats demonstrated significant levels of virus-neutralising antibodies compared to control animals [Kensil *et al.* 1991].

In another study using this preparation, cats were immunised three times at 0, 3 and 8 weeks and were challenged at 12 weeks by intraperitoneal inoculation of FeLV-A virus. After vaccination, all cats elicited anti-gp70 and anti-FOCMA antibody titres and two-thirds developed virus-neutralising antibodies. After challenge all cats produced a virus-neutralising immune response. None of the vaccinated cats developed viraemia compared to 3 of 4 control cats [Marciani *et al.* 1991]. Studies using this recombinant gp70 with Freund's adjuvant produced much weaker immune responses in this study. The gp70 vaccine with QS-21 adjuvant was subsequently marketed as Leucogen (Laboratoires Virbac, Nice, France).

A similar study was performed using the QS-21 adjuvanted vaccine using a schedule of 2 subcutaneous doses three weeks apart. Animals were challenged by intraperitoneal injection 2 weeks later. Of the vaccinated cats, 15% developed persistent viraemia, and 40% developed transient viraemia, which was cleared within 12 weeks of challenge, leaving 85% clear of viraemia. Latency of these cats was not measured in this experiment [Clark *et al.* 1991]. A field trial using pre-licensed vaccine at two 3 weekly doses were given to 1127 cats. Of 2234 doses, 150 post-vaccinal reactions were noted, consisting of small subcutaneous nodules at the vaccination site, or lethargy and anorexia for 24 hours post-vaccination [Clark *et al.* 1991].

A comparison of Leucogen, with Leukocell 2 or Leucat, both inactivated vaccines, showed that Leucogen was the only vaccine to produce significant protection against FeLV-A intraperitoneal challenge [Jarrett and Ganière 1996]. Five of the twelve kittens vaccinated with Leucogen became viraemic post-challenge while the rest developed virus-neutralising antibodies. All eight control animals were infected, one transiently. In addition, a study was conducted using an oronasal challenge of FeLV-A, -B and -C. In the control group, 5 of 6 kittens became persistently infected. p27 antigen was detected in 1 of 6 vaccinated kittens but unfortunately in these cases, no

virus isolation was performed. Two kittens developed virus-neutralising antibodies [Jarrett and Ganière 1996].

More recently, an experiment was carried out to investigate whether Leucogen could be combined with the routine live vaccine for feline herpesvirus, calicivirus and parvovirus (Feligen RCP, Virbac). The combination vaccine was found to give similar protection against FeLV as found by Jarrett and Ganière [1996], and protection from herpesvirus and calicivirus was enhanced by this combination. It was therefore concluded that these vaccines were both efficacious if coadministered [Guegen *et al.* 2000].

5.1.7 ISCOM vaccines

The ISCOM or immunostimulating complex is a novel way of presenting membrane proteins derived from enveloped viruses. Since its development the high immunogenicity of ISCOM formulations has led to the investigation of many such vaccines, including FIV and HIV-1 [Sjölander *et al.* 1996; Tijhaar *et al.* 1997].

The complex consists of a matrix combined with antigenic proteins of the pathogen. The matrix is made up of glycoside Quil A which forms micelles, allowing interaction between membrane proteins and matrix by hydrophobic interactions. This forms a cage-like structure of around 35nm diameter [Morein *et al.* 1984]. This system was used to create a vaccine for FeLV using gp70 and gp85 subunit purified from FL74 cell line supernatant. The purified subunits were shown to be shared by all subtypes of FeLV. When used in a vaccination regime, 66% of cats developed virus neutralising antibodies and all vaccinates were protected from viraemia. Of the control animals, 50% became viraemic post-challenge [Osterhaus *et al.* 1985].

This vaccine was further analysed by an experiment comparing the serological responses of ISCOM FeLV vaccine to vaccination with Leukocell. Pet cats were used and tested for positive or negative antibody titres to FeLV prior to the trial. During this study all animals remained healthy and no virus was detected. Almost every cat immunised with ISCOM produced increased antibody titres by the end of the

experiment. 81% of seronegative ISCOM cats displayed a rise in virus-neutralisation test compared to 6% in Leucocell group and 0% in control group. A similar pattern was observed in the seropositive cats. When the sera were analysed by immunoblotting, antibodies to gp70 and p15E were confirmed in the group vaccinated with ISCOM vaccine [Osterhaus *et al.* 1989]. However, antibodies to other proteins were also detected and the vaccine formulation was not highly purified. It would therefore be required to repeat this study using a purified version to confirm these results. Despite this, very few vaccines to FeLV have been found to induce virus-neutralising antibodies in this way [Jarrett, 1996], so these findings are of particular interest.

5.1.8 FeLV live virus vector vaccines

Several studies have utilised viral vectors to deliver FeLV antigens to the host. These have included recombinant vaccinia virus [Gilbert *et al.* 1987], a feline herpesvirus type 1 (FHV-1) [Wardley *et al.* 1992; Willemse *et al.* 1996] and a recombinant canarypoxvirus [Tartaglia *et al.* 1993].

The use of a vaccinia virus was found to provide low efficacy [Gilbert *et al.* 1987], but the FHV-1 vector has demonstrated greater success. As infection of FHV takes place via the mucous membranes, it was thought this could be advantageous for the delivery of FeLV genes as this pathogen also gains access to the host in this way. Wardley *et al.* [1992], used various combinations of FeLV *gag* and *env* in FHV-1 vectors administered oronasally and intramuscularly. Administration of both FeLV constructs by both of these routes stimulated complete protection against challenge, but only when an additional injection of baculovirus constructs containing the same FeLV genes was given. FeLV *gag* and *env* constructs administered alone did not produce the same protective effect [Wardley *et al.* 1992]. Another experiment described the use of two vaccinations of FHV-1 vector containing gp70/p15E of FeLV. The vaccine protected 3 of 4 cats from persistent viraemia where 5 of 6 cats developed viraemia in the control group. In terms of latency however, 3 vaccinates out of four demonstrated detectable levels of virus in the bone marrow [Willemse *et al.* 1996].

The use of canarypox vectors has been studied and an FeLV vaccine based on this work, Eurifel (Merial), became commercially available in 2002. An initial study used two recombinant vectors, one, ALVAC-FL expressed *gag* and *env* genes of FeLV-A, and the other, ALVAC-FL(dl IS) expressed *gag* and an *env* gene deleted of its putative immunosuppressive sequence in the TM (p15E) coding region. ALVAC-FL vaccine produced 100% protection from persistent viraemia following challenge without the production of virus-neutralising antibodies. ALVAC-FL(dl IS) produced only 50% protection from viral challenge [Tartaglia *et al.* 1993].

Table 5-3 FeLV vaccines commercially available in the UK and worldwide

Vaccine (Manufacturer)	Vaccine type	Subgroups of FeLV	FOCMA included	UK
Leukocell 2 (Pfizer)	Produced from infected cell culture- mixed protein subunits. Inactivated and adjuvanted	A, B and C	Yes	Yes
Leucat / VacSYN (Rhône Merieux/Synbiotics)	Inactivated whole virus. No adjuvant	A, B and C	Yes	No
Fel-O-Vax (Fort Dodge).	Inactivated adjuvanted whole virus	A	No	No
Fevaxyn (Solvay-Dulphar/Fort Dodge)	Inactivated adjuvanted whole virus	A and B	No	Yes
Leucogen/ Genetivac/Nobivac FeLV (Virbac/Mallinckrodt/Intervet)	Purified, recombinant non-glycosylated gp70. Adjuvanted	A	No	Yes
Eurifel (Merial)	Recombinant canarypox vector containing <i>gag</i> and <i>env</i> genes	A	No	Yes

5.1.9 Immunisation using a FeLV DNA vaccine

5.1.9.1 Vaccine design and schedule

To date there is only one published study investigating the use of a DNA vaccine for FeLV [Hanlon *et al.* 2001]. This study used a combination of two plasmids, one encoding *gag/pol* and the other encoding *envA* of FeLV-A, each in a vector derived from pCI-neo (Promega). The efficacy of this vaccine was studied alone and with various combinations of cytokine genes as adjuvants to the vaccine.

Five groups of 10-12 week old cats were used and immunised three times at 2-week intervals with 100 µg of each vaccine construct. Animals were then challenged 3 weeks later with an intraperitoneal injection of 2×10^5 f.f.u. of FeLV-A/Glasgow-1 virus diluted in endotoxin-free PBS [Hanlon, 1999; Hanlon *et al.* 2001]. The vaccine groups were arranged as follows:

Table 5-4 Immunisation groups of FeLV DNA vaccine trial [Hanlon *et al.* 2001]

Group	Vaccine constructs	Number of cats
1	Vaccine alone	6
2	Vaccine + IFN γ	5
3	Vaccine + IL-12	6
4	Vaccine + IL-12 + IL-18	6
5	Empty vaccine vector	6

Animals were then blood sampled every three weeks until euthanasia, 16 weeks post-challenge, where a sample of bone marrow was taken. Plasma was analysed for the presence of p27 antigen, infectious virus and neutralising and non-neutralising antibodies. This was to detect the presence of viraemia and determine the immune response to the virus. Testing of cultured bone marrow cell lysate and supernatants for p27 antigen and virus respectively was also performed to determine the presence of latent infection in animals.

5.1.9.2 Protection against FeLV viraemia

When the virus isolation (VI) results of groups 1 (vaccine alone) and 5 (negative control) were compared, there was no statistical difference found between them. At euthanasia 3 of 6 control cats were viraemic whereas 2 of 6 cats from the vaccine alone group were viraemic. This showed that immunisation with the DNA vaccine alone did not provide significant protection against FeLV challenge.

Of the five cats in group 2 (vaccine + IFN γ), two animals were viraemic at euthanasia. When these are compared with the control and vaccine alone group it showed that this vaccine combination did not produce immune protection and IFN γ did not act as an effective vaccine adjuvant. A similar conclusion can be drawn from the vaccine + IL-12 group where 4 of 6 cats were viraemic at euthanasia. This suggests that IL-12 might even have inhibited vaccine efficacy.

In contrast, cats in group 2 (vaccine + IL-12 + IL-18) demonstrated significant protection from viral challenge. All of the cats were free of viraemia at euthanasia and significantly fewer were viraemic compared to control cats at 3, 6 and 9 weeks post-challenge. It can be concluded, therefore, that the combination of IL-12 and IL-18 had the potential to act as an adjuvant to this DNA vaccine.

5.1.9.3 Protection against persistent and latent FeLV infection

Unfortunately there were insufficient animals in each group to specifically analyse the efficacy of vaccines in protection against latency. Analysis of the sum of viraemic and latently infected cats in each group, however, allowed further comparison of vaccine groups.

A similar pattern was found as with protection against viraemia, in that neither the vaccine alone, vaccine + IL-12 nor vaccine + IFN γ offered significant protection against viraemia and latency compared to the control group. However, only one cat in the vaccine + IL-12 + IL-18 group carried a latent infection which was only detected after subculture of bone marrow supernatant. When compared with five control cats carrying both persistent and latent infection, this again is a statistically significant

result. This suggests that this vaccine combination protected cats against both persistent and latent infection.

5.1.9.4 Analysis of antibody levels and immune responses elicited during trial

DNA immunisation induced neither neutralising nor non-neutralising antibodies at detectable levels between vaccination and viral challenge. Once cats were exposed to virus, protected cats tended to demonstrate a significant neutralising antibody titre whereas infected cats failed to elicit a FeLV-neutralising antibody response.

This display of immune protection in the absence of antiviral antibodies led to a study of FeLV-specific CTL responses in these cats. It was found that vaccine-protected cats had higher levels of specific-CTLs in the peripheral blood and lymphoid organs compared to the unvaccinated persistently infected cats. Transiently infected cats in this study also had higher specific-CTL responses [Flynn *et al.* 2000a].

5.1.10 Design of current trial

The aim of this work was to investigate further the data produced from this previous DNA vaccine study [Hanlon *et al.* 2001]. Preliminary work described in chapters 3 and 4 involved the cloning of refined constructs for IL-12 and IL-18 to be used in the DNA vaccine trial. Feline flexi-IL-12 comprised sequences derived from IL-12 p40 and signal deleted-p35 linked by a synthetic polypeptide linker sequence [Anderson *et al.* 1997]. ILRAP-IL-18 was produced by cloning the signal sequence of human IL-1 β receptor antagonist protein (ILRAP) as a secretory signal for mature feline IL-18 [Wingren *et al.* 1996]. As mentioned in the respective chapters, each of these constructs has potential advantages over those used in the original trial. *In vitro* expression and bioactivity of flexi-IL-12 and ILRAP-IL-18 was demonstrated and in addition, secretion of ILRAP-IL-18 from transfected cells was detected by Western blot and bioactivity of culture fluids.

Design of the new trial focussed on establishing whether protection induced by the DNA vaccine, IL-12 and IL-18 was due to either cytokine alone, or a synergistic

effect of IL-12 and IL-18 [Hanlon *et al.* 2001]. The experiment was designed comprising the following groups; DNA vaccine alone, vaccine + IL-12 and IL-18, vaccine + IL-12 and vaccine + IL-18. In addition to these groups, two control groups were included. One group was inoculated with both cytokines and given the equivalent amount of empty pCI-neo vector as DNA vaccine. This was to determine firstly if the cytokines alone produce an immunostimulatory effect, and secondly to establish whether non-specific DNA affects the immune response. Finally a negative control group was injected with vaccine diluent alone (endotoxin-free PBS).

The final aim of this experiment was to establish if the vaccine was able to protect cats against a more natural viral challenge. Rather than an intraperitoneal challenge of 2×10^5 f.f.u. used previously, a total oronasal dose of 4×10^6 f.f.u. was administered in four equal doses of 10^6 f.f.u. on days 0, 2, 4 and 8. This method of challenge was found previously to be sufficient to produce 82% persistent infection in kittens of a similar age [Harbour *et al.* 2002].

5.2 MATERIALS AND METHODS

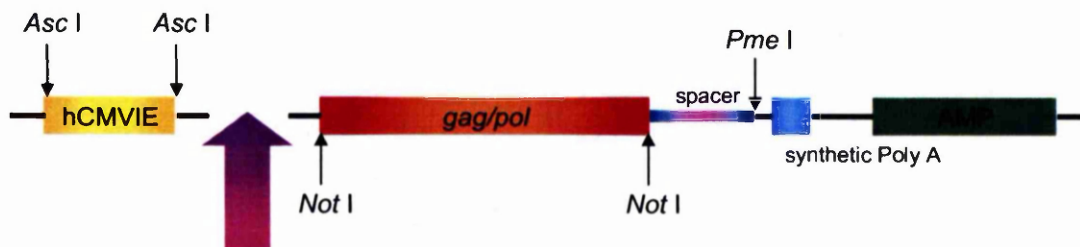
5.2.1 Construction of FeLV DNA vaccine

Dr Derek Bain constructed the plasmid backbone for FeLV vaccine plasmids using the commercial plasmid pCI-neo (Promega). A mammalian expression series of bicistronic vectors pUSE, pUSE1⁻ and pUSE2⁻ were cloned using PCR and recombinant DNA technology. Included in these vectors were unique restriction sites for *Asc* I, *Pac* I, *Pme* I and *Not* I. These sites allowed efficient cloning of reporter genes, promoters and insertion of antigen-encoding gene into the empty plasmid backbone.

The FeLV DNA vaccine consisted of a combination of two pUSE1⁻ plasmids containing a CMV-IE promoter. One plasmid contained the gene encoding FeLV *gag/pol* and the other contained the gene encoding *env* of FeLV-A.

5.2.1.1 Construction of pUSE1⁻CMVT(*gag/pol*) and pUSE1⁻CMVT(*envA*)

Figure 5-1 Structure of pUSE1⁻CMVT(*gag/pol*)

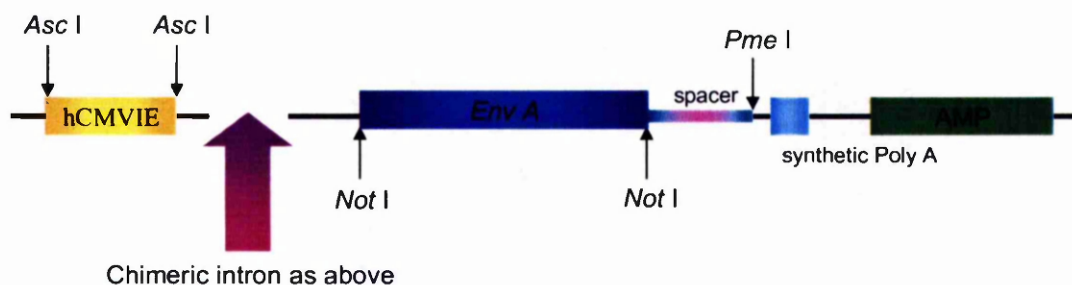


Chimeric intron: 5' is donor site from the first intron of the human beta-globin and the branch and 3' -acceptor of an immunoglobulin gene heavy chain variable region.

The structure of the plasmid is shown in the figure 5.1. The construct consists of FeLV-A/Glasgow-1 *gag/pol* gene and a CMV promoter cloned into the *Not* I site of pUSE1⁻CMVT to produce pUSE1⁻CMVT(*gag/pol*).

Similarly, the *env* gene from FeLV-A/Glasgow-1 was cloned into pUSE1⁻ vector with a CMV promoter to produce pUSE1⁻CMVT(*envA*) as shown in figure 5.2.

Figure 5-2 Structure of pUSE1⁻CMVT(*gag/pol*)



5.2.1.2 Expression of DNA vaccine plasmids using fixed cell immunofluorescence

293T cells were seeded into 25 cm² flasks and incubated for 24 hours at 37°C with 5% CO₂ until approximately 40% confluent. Transfections of pUSE1⁻CMVT(*gag/pol*), pUSE1⁻CMVT(*envA*) and pCI-neo plasmid vector (Promega) were performed using LipofectAMINE Reagent (Invitrogen Life Technologies) according to the instructions of the manufacturer. For each transfection, 3 µg of DNA was used and flasks were incubated for 24 hours at 37°C with 5% CO₂. Each flask of transfected cells was trypsinised using 1ml trypsin-EDTA, suspended in fresh medium, centrifuged at 1000 rpm and then resuspended in tissue culture medium. Transfected cells were then transferred to the CC2 treated Lab Tek II chamber slide system consisting of 8-well glass slides (Scientific Laboratory supplies, UK). Three wells were prepared for each transfection using 3 x 10⁴ cells per well in 200 µl culture medium. Alongside transfected cells, duplicate wells of FEA cells and FEA cells infected with FeLV-A/Glasgow-1 virus (FeLV-A cells) were prepared as negative and positive controls respectively. All slides were then incubated for a further 24 hours as above.

Cell medium was removed from the wells and the cells were gently washed 3 times with PBS. Cells were fixed by adding 100 µl methanol to each well and incubating for 1 hour at room temperature. The methanol was removed and cells were washed twice with PBS. Cells were then incubated with either 6-15 monoclonal antibody specific for FeLV gp70 antigen to detect expression of pUSE1⁻CMVT(*envA*), PF12J-10A monoclonal antibody specific for FeLV p27 antigen to detect pUSE1⁻CMVT(*gag/pol*) expression or PBS buffer as negative antibody control. These primary antibodies were

used at a 1:50 dilution in PBS and all cells were incubated for 1 hour at 37°C with 5% CO₂. Wells were prepared as follows:

Table 5-5 Transfected cell lines and antibodies used for immunofluorescence

Well	Transfected cell line	Transfected DNA	Antibody	Antigen recognition
1	293T	pUSE1 ⁺ CMVT(<i>gag/pol</i>)	PF12J-10A	CA (p27)
2	293T	pUSE1 ⁺ CMVT(<i>gag/pol</i>)	6-15	SU (gp70)
3	293T	pUSE1 ⁺ CMVT(<i>gag/pol</i>)	PBS	None
4	293T	pUSE1 ⁺ CMVT(<i>envA</i>)	PF12J-10A	CA (p27)
5	293T	pUSE1 ⁺ CMVT(<i>envA</i>)	6-15	SU (gp70)
6	293T	pUSE1 ⁺ CMVT(<i>envA</i>)	PBS	None
7	293T	pCI-neo	PF12J-10A	CA (p27)
8	293T	pCI-neo	6-15	SU (gp70)
9	293T	pCI-neo	PBS	None
10	FEA	none	PF12J-10A	CA (p27)
11	FEA	none	6-15	SU (gp70)
12	FeLV-A	none	PF12J-10A	CA (p27)
13	FeLV-A	none	6-15	SU (gp70)

Following incubation, primary antibody was removed and all wells were washed 3 times with PBS. A secondary antibody was prepared in PBS using anti-mouse IgG (whole molecule) FITC conjugate (Sigma) at a 1:250 dilution and 0.5% Evans blue at a dilution of 1:1000. A volume of 100 µl of this solution was added to all wells and slides were incubated for 1 hour at 37°C with 5% CO₂. After this incubation, the secondary antibody was removed and cells were washed 3 times in PBS. The wells were dried and kept in the dark until viewing under the UV microscope. Images of cells undergoing fluorescence were captured using Fujichrome 400 film (H.A. West, Clydebank, UK) in a 35 mm camera attached to the microscope.

5.2.1.3 *In vitro* expression of *gag/pol* constructs using Western blot analysis

Previous work had failed to elicit *in vitro* expression of pUSE1⁺CMVT(*gag/pol*) and pUSE1⁺CMVT(*envA*) by Western blot analysis [Hanlon, 1999]. An experiment was therefore performed to establish whether the level of FeLV CA (p27) protein expression elicited by the *gag/pol* gene was affected by the plasmid expression vector used. Therefore *gag/pol* was cloned into the commercial expression vector pCI-neo (Promega) and the protein expressed from cell transfections were compared by

Western blot analysis. The same cloning procedure was also performed using the *envA* gene but Western blot analysis of SU (gp70) protein expression was unsuccessful and will not be detailed here.

5.2.1.4 Cloning of pCI-neo(*gag/pol*)

DNA encoding FeLV *gag/pol* was excised from pUSE1⁺CMVT(*gag/pol*) using a *Not* I bulk digest. A *Not* I bulk digest of pCI-neo expression vector opened the vector for insertion of FeLV *gag/pol*. Ligation of digested vector and *gag/pol* fragment produced pCI-neo(*gag/pol*). This ligation was transformed into MAX Efficiency STBL2 competent cells (Invitrogen) using the instructions of the manufacturer and bacterial colonies were selected into 3 ml of LB culture medium. These were incubated overnight at 37°C in an orbital incubator. DNA was prepared from these cultures using Qiaprep Miniprep Kit, (Qiagen) and positive clones were verified by restriction digestion. A positive clone was then sequenced using the ABI Prism system. T7 and M13 reverse primers were used to verify the sequence and orientation of the inserted DNA.

5.2.1.5 Transfection and harvesting of pUSE1⁺CMVT(*gag/pol*) and pCI-neo(*gag/pol*)

293T cells were seeded into 75 cm² flasks and incubated for 24 hours at 37°C with 5% CO₂ until approximately 40% confluent. Transfections of pUSE1⁺CMVT(*gag/pol*), pCI-neo(*gag/pol*) and pCI-neo plasmid vector (Promega) were performed using LipofectAMINE Reagent (Invitrogen Life Technologies) following the manufacturer's instructions. Flasks were then incubated for 48 hours at 37°C with 5% CO₂.

Supernatant fluids were decanted from the transfections and centrifuged at 1000 rpm for 5 minutes to remove any cell debris. The supernatant was then dispersed in aliquots and stored at -20°C until further use. Cells were removed from the bottom of the flask using a sterile scraper, suspended in 2 ml PBS and centrifuged at 1000 rpm for 5 minutes to pellet the transfected cells. The supernatant was discarded and cells were resuspended in 100 µl lysis buffer (1 M Tris pH 7.4, 1.4 M NaCl, 10 mM EDTA, 10% Triton X-100, 1% SDS, 5% deoxycholate, 10% Aprotinin) by pipetting

up and down gently. Cellular debris was removed by centrifugation for 30 seconds at 14000 rpm and the lysate supernatant was stored at -20°C until further use.

5.2.1.6 Western blot analysis of pUSE1⁺CMVT(*gag/pol*) and pCI-neo(*gag/pol*)

A 15% SDS-PAGE gel was prepared using pUSE1⁺CMVT(*gag/pol*), pCI-neo(*gag/pol*) and pCI-neo supernatants and cell lysates. In the initial experiment, 20 µl of each neat sample with 5 µl protein loading buffer was used. A positive control, consisting of FeLV-infected F422 cell lysate, was kindly supplied by Mathew Golder, University of Glasgow. A 5 µl sample of 1:20 F422 lysate dilution was run alongside transfection samples.

Protein from the gel was transferred to a PVDF membrane using a semidry immunoblotter as described in 2.2.11. The membrane was exposed to a 1:80 dilution of PF12J-10A mouse monoclonal antibody specific to FeLV p27 antigen. The secondary antibody was anti-mouse HRP conjugate, used at a concentration of 1:2000.

On demonstration of *in vitro* expression of p27 antigen in both pUSE1⁺CMVT(*gag/pol*) and pCI-neo(*gag/pol*), Western blot analysis was repeated using 1:2 serial dilutions of each transfection supernatant to detect any difference in protein expression between the two expression vectors.

5.2.2 Preparation of DNA vaccine and cytokine DNA for immunisation

Once the plasmid vector for the DNA vaccine had been selected and the bioactivity of flexi-IL-12 and ILRAP-IL-18 constructs verified, the vaccine components were prepared using large-scale DNA purification techniques.

Glycerols of pUSE1⁺CMVT(*gag/pol*), pUSE1⁺CMVT(*envA*), flexi-IL-12, ILRAP-IL-18 and pCI-neo were streaked out onto separate LB-agar plates using a sterilised platinum wire and incubated overnight at 37°C. A single colony for each construct was selected and grown in 8 ml LB medium supplemented with 100 µg/ml ampicillin

in a 15 ml Falcon tube. Tubes were incubated at 37°C with vigorous shaking at 225 rpm for 6-8 hours. This starter culture was then inoculated into 2.5 litre LB medium with ampicillin, which was incubated overnight at 37°C with vigorous shaking. Five 2-litre glass flasks, each containing 500 ml of culture, were used for this incubation.

Preparation of DNA from this large-scale culture was carried out using the Qiagen plasmid Endofree Giga Kit, (Qiagen) under the instructions of the manufacturer. This kit is designed to purify up to 10 mg of low endotoxin, high-copy plasmid DNA in each batch. Endotoxins, or lipopolysaccharides make up a large proportion of the cell membrane of Gram-negative bacteria such as *E. coli*. It is crucial that endotoxin-free DNA is used in *in vivo* experiments as endotoxins can produce endotoxic shock syndrome, fever, and activation of the complement cascade in both animals and humans [Vukajlovich *et al.* 1987].

In order to prevent contamination of DNA preparations with endotoxin, pyrogen-free Rainin Greenpak filter tips (Anachem) were used and DNA was stored in 2 ml endotoxin-free Eppendorf tubes (Fisher Scientific). In addition, endotoxin-free water (Sigma) was used when necessary and DNA was resuspended in endotoxin-free PBS (Biowhittaker UK Ltd.). All tubes and pipettes used in the procedure were pyrogen-free.

The DNA pellet was generally resuspended in 1 ml endotoxin-free PBS and incubated overnight at 37°C in order to produce complete dissolution of the DNA. Each batch of DNA was then stored at -20°C prior to quantification, sequencing and endotoxin testing.

5.2.2.1 Quantification and sequencing of vaccine and cytokine DNA

Restriction digests of each batch of plasmid DNA were carried out as a preliminary measure to verify construct inserts. Quantification of DNA was achieved by spectrophotometry and by comparison to standards on gel electrophoresis. Antigen or cytokine sequence in each batch of DNA was verified either partially or in full by sequencing using ABI Prism using appropriate primers.

5.2.2.2 Endotoxin testing of vaccine and cytokine DNA

Each batch of DNA was tested for endotoxin using the *Limulus* amoebocyte lysate (LAL) assay. Batch samples were sent to Biowhittaker Europe, Verviers, Belgium for testing.

The concept behind this assay is the ability of endotoxin to initiate clotting of *Limulus polyphemus* haemolymph. In its simplest form, the time taken for DNA samples to form a solid clot when mixed with LAL enzyme and substrate is a measure of endotoxin present. This assay has been developed by replacement of clotting protein with a synthetic peptide. This peptide is covalently attached to para-nitroanilide, which on contact with endotoxin is cleaved and produces a colour change. This change is proportional to the amount of endotoxin present and is measured by spectrophotometry at 405 nm [Novitsky, 1983]. The acceptable level of endotoxin allowed in each batch was taken as <50 EU/mg of DNA [Schorr *et al.* 1995].

5.2.3 Virus used for challenge

The virus used for challenge was kindly provided by Mathew Golder. Wild-type FeLV-A/Glasgow-1 virus was cultured in the FEA cell line [Jarrett *et al.* 1973]. Before use, the virus batch was assayed using the QN10 cell line. Duplicate tests were carried out of ten-fold dilutions of virus from 10^{-4} to 10^{-6} and the mean of these values was taken as the viral titre. For the trial, batch 56 had a titre of 4.2×10^6 f.f.u./ml. The virus was stored at -80°C and thawed immediately before use.

5.2.4 Design of DNA vaccine trial

5.2.4.1 Maintenance of experimental animals

Thirty-six specific pathogen free (SPF), 11-13 week old kittens were acquired from a commercial breeding establishment and housed in the isolation unit at the University of Glasgow. They were arranged randomly on arrival, renamed L1-L36 and split into 6 rooms of 6 cats. Each experimental group was housed in a separate room within the isolation unit. Cat L7 was euthanased 11 weeks into the trial leaving one room housing 5 cats. L7 had displayed sudden onset anorexia, weight loss and ataxia and was removed from the trial and euthanased. On post mortem, a chronic fibrosing interstitial nephritis was identified with an accumulation of crystals in the renal tubules. It was concluded that uraemia from renal disease had probably caused the neurological signs which were displayed.

Animals were fed according to weight with a commercial diet of canned and dried food. All procedures were performed following Home Office regulations.

5.2.4.2 Vaccination groups

Each group of cats was immunised with a different vaccine combination as follows:

Table 5-6 Immunisation groups used for current DNA vaccination trial

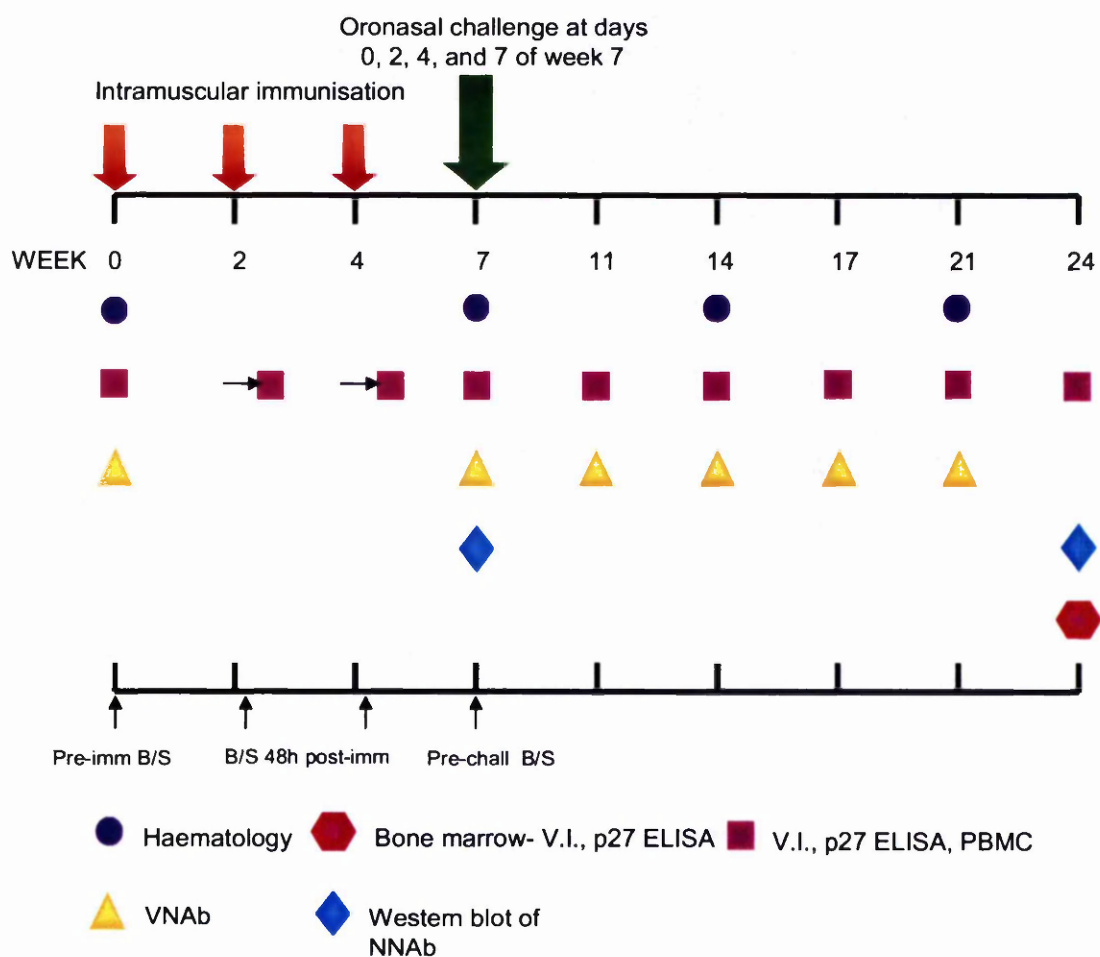
Group	Cats	Vaccine combination
1	L1 - L6	DNA vaccine + flexi-IL-12 + ILRAP-IL-18
2	L8 - L12	DNA vaccine + flexi-IL-12
3	L13 - L18	DNA vaccine + ILRAP-IL-18
4	L19 - L24	DNA vaccine
5	L25 - L30	pCI-neo vector + flexi-IL-12 + ILRAP-IL-18
6	L31 - L36	PBS

The DNA vaccine in each case consisted of an equal combination of pUSE1⁻CMVT(*gag/pol*) and pUSE1⁻CMVT(*envA*).

5.2.4.3 Immunisation schedule

A diagram of the vaccination schedule is shown in figure 5.3.

Figure 5-3 Vaccination schedule: parameters tested at each timepoint



Once the animals had been separated into rooms they were allowed to acclimatise to their environment for 2 weeks before commencement of the trial. Endotoxin-free vaccine DNA was prepared as described previously in 5.2.2. Cats were immunised three times at two-week intervals at weeks 0, 2 and 4. Each immunisation comprised 100 µg of each vaccine component suspended in 200 µl endotoxin-free PBS (Biowhittaker UK Ltd.). Cats were manually restrained and the vaccine was administered intramuscularly using a sterile insulin syringe and needle. The first and

third vaccines were injected into the left quadriceps femoris muscle and the second vaccine into the right quadriceps femoris. For 24 hours after each immunisation, kittens were carefully monitored and no adverse local or systemic reactions were observed.

5.2.4.4 Viral challenge

Kittens were challenged with wild type FeLV-A/Glasgow-1 virus which had been previously assayed as mentioned earlier. They were challenged 3 weeks after the final immunisation at days 0, 2, 4 and 8 as performed in a previous study [Harbour *et al.* 2002]. Each challenge consisted of 1×10^6 f.f.u. diluted to a total volume of 1 ml in FEA cell medium, producing a total challenge to each kitten of 4×10^6 f.f.u. To mimic natural transmission of FeLV via the saliva, virus was administered to the cats oronasally, 0.5 ml into the mouth and 0.25 ml into each nostril. Any cats that became persistently infected, usually after an incubation period of 3-4 weeks, would subsequently be a source of infection to other cats in the same room exposing them to an additional natural challenge. Again, cats were closely observed over the challenge period and no adverse local or systemic reactions were observed.

5.2.4.5 Blood sampling

Blood sampling of kittens was performed in accordance with the guidelines of the Home Office. Home Office regulations stipulate that the maximum volume of blood that can be taken per 100 g bodyweight is 0.5 ml every two to three weeks. With this maximum volume in mind, blood for screening was taken as follows:

Table 5-7 Quantities of blood taken during DNA vaccination trial

Blood sample	Week	Amount of blood (ml)				
		p27, VI, VNAb, NNAb* (Heparin)	Haematology (EDTA)	PBMC (Alsevers)	PBMC (Citrate)	Total
pre-immun	0	1	0.5	1		2.5
post-immun	2	1		2		3
post-immun	4	2		2		4
pre-challenge	7	2	0.5	3		5.5
post-challenge	11	2		4		6
post-challenge	14	2	0.5	5		7.5
post-challenge	17	2		6		8
post-challenge	20	2	0.5	7		9.5
euthanasia	23	2			1	3

- p27 - FeLV p27 ELISA
- VI - FeLV virus isolation
- VNAb - FeLV virus neutralising antibodies assay
- NNAb* - FeLV non-neutralising antibodies assay; this was performed at viral challenge and at euthanasia

Cats were manually restrained and the jugular or cephalic vein area was shaved and prepared using Hibitane solution. Where necessary, cats were anaesthetised using a 30% dose regimen of a xylazine (Rompun, Bayer plc, Bury St. Edmunds, UK) and ketamine (Ketaset, Willows Francis Veterinary, Crawley, UK) combination according to the instructions of the manufacturer. Blood samples at euthanasia were collected from anaesthetised cats using intracardiac puncture.

5.2.4.6 Screening of blood samples

Screening of blood for FeLV p27 antigen, virus isolation and antibodies

At every sampling point in the trial, heparinised plasma was screened for infectious virus by virus isolation (VI) and p27 antigen by ELISA. Antibodies produced to FeLV, both virus neutralising (VNAb) and non-neutralising (NNAb), were measured in each cat as shown in figure 5.3. Blood was collected in 2 ml heparin tubes and centrifuged at 2000 rpm for 5 minutes. The plasma was removed, aliquoted and stored at -70°C until further use.

The purpose of pre-immunisation screening was to establish the FeLV status of cats and ensure no prior exposure to the virus. Of particular interest at this point was whether vaccination stimulated the production of anti-FeLV antibodies.

Sampling post-challenge was performed every three weeks until euthanasia. This was to establish the viral status of cats and to analyse any immune responses stimulated in response to viral challenge. These data were then used to compare the ability of different vaccine combinations to induce protection against exposure to FeLV. Details of p27 ELISA, VI, VNAb and NNAb assays are described in detail later in 5.2.5.

Screening of blood for haematology

At various points in the experiment, blood was collected into 2 ml potassium-EDTA tubes (Bibby Sterlin Ltd., Stone, UK) to establish the haematological status of the cats. Cells were counted using an automated cell counter (Abbott, CELL-DYN 3500, Abbott Diagnostics Division, Abbott Park, IL 60064 USA) and differential cell counts were carried out manually using May-Grunwald-Giemsa stained smears.

The parameters measured were as follows: red blood cell count (RBC), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), and white blood cell count (WBC). Proportional representation of white blood cells into neutrophils, lymphocytes, monocytes eosinophils and basophils was also established. All of these levels were compared with normal parameters to confirm the health status of the cats.

PBMCs collected from Alsevers solution

At each sampling point, PMBCs were extracted from blood collected in a 15 ml Falcon tube at a 1:1 ratio with Alsever's solution (Diagnostics Scotland, Edinburgh). Alsever's is a sterility tested solution with anticoagulant and preservative properties containing citrate and glucose. This blood/Alsevers mix was laid over an equal volume of Ficoll-Paque PLUS lymphocyte isolation solution (Amersham Pharmacia Biotech AB, Sweden) contained in a 50 ml Falcon tube. Tubes were centrifuged without braking at 1500 rpm for 15 minutes. The PBMC interface layer was removed

and diluted in 20 ml RPMI 1640 medium with L-glutamine. Cells were pelleted by centrifugation at 1500 rpm for 15 minutes without braking and the supernatant removed. The cell pellet was gently resuspended in freeze medium consisting of 90% heat inactivated FCS and 10% DMSO (Analar, BDH Ltd) and transferred to a 2 ml cryogenic vial. These tubes were then stored at -70°C until further use.

PBMCs collected from sodium citrate anticoagulant

PBMCs were also collected from sodium citrate blood at the final sampling point of the trial. These tubes were centrifuged at 2000 rpm for 5 minutes and the plasma removed and stored at -70°C. A small sample of the buffy coat was then transferred into an Eppendorf tube, centrifuged for 5 minutes at 2000 rpm to remove any plasma and stored at -70°C.

5.2.4.7 Collection and analysis of bone marrow

At the end of the trial, cats were anaesthetised using an intramuscular injection of xylazine and ketamine and euthanased by an intracardiac injection of pentobarbitone (Euthatal). All cats were euthanased except persistently infected cats in the control group, L31, L33, L34 and L35. These four cats were retained for an immunotherapy experiment described in chapter 6. Bone marrow was collected from all euthanased cats and bone marrow biopsy was performed on the remaining 4 cats in order to establish the presence of latent FeLV infection.

In euthanased cats, the femur was removed and bone marrow was taken aseptically using bone cutting forceps. Each sample was collected in a 5 ml bottle containing 3 ml bone marrow transport medium. This medium consisted of Alpha Minimal Essential Medium (Invitrogen), supplemented with 10% FCS, 2 mM L-glutamine, 400U/ml penicillin/streptomycin, 10^{-6} M hydrocortisone succinate (AFC10) and 50 IU/ml preservative-free heparin (Pularin).

The four cats requiring bone marrow biopsy were anaesthetised using intravenous injection of alphaxelone/alphadolone acetate (Saffan, Pitman-Moore Ltd., Crewe, UK) at a dose recommended by the manufacturer. The left gluteal area was shaved

and prepared aseptically. The inter-trochanteric fossa was located and a small incision made over this region using a sterile scalpel. A bone marrow aspirate was taken using a 10 ml sterile syringe and a sterile disposable 18G Illinois Sternal/Iliac bone marrow aspiration needle (Pharmaseal, Allegiance Healthcare Corporation, USA.). Each aspiration was collected in bone marrow transport medium as described above. The preparation and culture of bone marrow samples is explained in detail in 5.2.5.5.

5.2.5 Feline leukaemia virus screening

5.2.5.1 FeLV p27 antigen ELISA

Greiner high-binding wells were used for this assay. Each well was prepared by coating with a mix of 2 purified murine monoclonal antibodies, MCA 0022 and MCA 0024, raised to p27 antigen. The protein concentration of these antibodies was approximately 1 mg/ml suspended in PBS and the preparation contained azide. Antibodies were diluted 1:300 in coating buffer and added immediately to wells. Any delay in this addition brought about adhesion of antibody to the container in which it was diluted. Plates were covered and incubated on an orbital platform overnight -4°C and were washed 5 times in PBS/0.05% Tween.

Biotinylated sheep anti-p27 antibody was diluted at 1:4000 in PBS and 80 µl was added to each well. Twenty microlitres of sample or positive or negative control was then added to wells. The plates were covered and incubated at 37°C for 45 minutes with continuous shaking. A solution of streptavidin-alkaline phosphatase conjugate (Harlan) was prepared at a dilution of 1:4000 in PBS and 100 µl was added to each well and wells were incubated with shaking at 37°C for 30 minutes. Plates were washed 5 times as before and 100 µl phosphatase substrate was added to each well. Substrate was prepared by dissolving a 5 mg p-nitrophenyl phosphate tablet in 5 ml of alkaline phosphatase buffer (5.36 g diethanolamine, 2.92 g NaCl, 0.508 g MgCl in 500 ml dH₂O, pH 9.5.). Wells were covered and incubated on an orbital shaker at 37°C for 30 minutes. The reaction was stopped by the addition of 50 µl 0.5 M NaOH to each well. The optical density of each well was then read immediately using a spectrophotometer (Ascent-AFVU) at 405 nm wavelength. A sample was taken as

positive if it gave an OD reading of over 1.5 times the value of the negative control reading for that batch of samples.

5.2.5.2 Virus isolation technique

This method of virus isolation was described by Jarrett and Ganière, [1996]. QN10 cells were grown in 22 mm diameter, 12-well cluster plates. 5×10^4 cells in 1 ml culture medium were added to each well, supplemented with 4 µg/ml polybrene and incubated for 24 hours at 37°C with 5% CO₂. At this time, 0.2 ml of sample was added to each well using separate pipettes to avoid cross-contamination and plates were incubated for 2 hours at 37°C. The inoculum was then removed and replaced with 1.5 ml fresh medium and the plates were incubated for 3-5 days. Cells were examined for evidence of morphological transformation. Positive control wells consisted of ten-fold dilutions of FeLV-A. A negative control well using serum from a FeLV-A-free SPF cat was also included.

Samples with morphological transformation were diagnosed as positive for virus at this point whereas negative samples were subcultured. The reason for the subculturing step is that if samples contain low levels of FeLV there may be insufficient virus to produce a visual morphological effect. The wells of these samples and controls were trypsinised using 250 µl of trypsin/versene mixture and left until the cells had detached. Cells were resuspended in 1 ml culture medium and added to a 5 cm plate containing 4 ml fresh medium. These plates were incubated for 3-4 days as before, viewed for morphological transformation and samples were scored positive or negative on this basis. Transfer of QN10 cells to 5 cm dishes at subculturing allows a faster rate of cell division. This promotes visualisation of transformation as FeLV replicates only in actively dividing cells.

5.2.5.3 Assay for FeLV virus neutralising antibodies (VNAb)

Measurement of VNAb to FeLV in plasma was performed using a focus reduction assay previously described by Jarrett and Ganière [1996]. For each plasma sample, 4 wells were prepared at 4×10^4 QN10 cells per well in 12 well cluster plates. These cells were suspended in 1 ml of culture medium and were incubated overnight at 37°C with 5% CO₂.

Samples were diluted in Leibovitz medium from 1:4 to 1: 256 and 50 µl of dilutions were aliquoted onto 96 well plates. 50 µl of FeLV-A at a concentration of 4×10^2 f.f.u./ml was added to each well and incubated for 6 hours at 37°C with 5% CO₂. Of the sample dilutions, 25 µl of 1:4, 1:8, 1:16 and 1:32 dilutions were each added to a QN10 cell culture and incubated for 3-4 days at 37°C. The rest of the sample dilutions were stored at -70°C.

After incubation, QN10 cells were examined for the presence of foci of transformation, which allowed quantification of infectious virus levels. The reciprocal of the plasma dilution which reduced the focal count of FeLV by 75%, was taken as the titre of antibody present when compared with a virus control incubated without plasma.

5.2.5.4 Assay for FeLV virus non-neutralising antibodies (NNAb)

The presence of non-neutralising antibodies was detected by Western blot analysis against complete viral lysate.

An SDS-PAGE gel was prepared as described in 2.2.10. Prior to running the gel the concentration of p27 in the viral lysate was estimated by comparing the intensity of p27 protein to an ovalbumin control of known concentration on a Coomassie blue stained gel. The total viral protein was then calculated as p27 accounts for about 30% of total protein in the sample. Approximately 170 µg of viral protein was added to 25 µl of 3 x protein sample loading buffer and protein was separated on an SDS-PAGE gel along with protein molecular weight standards.

Protein was transferred to a nitrocellulose membrane using electroblotting equipment at 100 V for 1 hour and the membrane was removed and washed in TBS. It was then incubated on an orbital shaker for 3-4 hours in TBS/2% Marvel solution to block non-specific binding sites. The membrane was washed in PBS/0.05% Tween 20 for 2 hours, air dried at room temperature by placing on 3 MM paper between folded parafilm, and stored at -20°C in a sealed freezer bag until further use.

When required, the membrane was thawed at room temperature and cut into vertical strips each around 1 mm in width. Identical strips were cut for each cat serum sample as well as positive and negative controls. Individual plasma samples were incubated with a separate strip to identify any antibodies to FeLV proteins.

Any antibody bound to protein on the membrane was visualised using the streptavidin peroxidase method. In this protocol, strips were placed in individual compartments of multi well dishes and solution A was prepared using 0.8 g Marvel in 20 ml TBS with 100 µl Tween 20. A 20 µl plasma sample from each cat was made up to 200 µl with solution A containing 1 mM EDTA and added to a strip within a well. This was incubated at room temperature for 2 hours (or overnight at 4°C) and the strips were washed 3 times in PBS/0.05% Tween. Biotinilated protein A (Amersham Life Sciences) was diluted in solution A at a concentration of 4 µl/ml. 200 µl was added to each strip and incubated with gentle rocking for 1 hour at room temperature. Strips were washed again in PBS/0.05% Tween and 200 µl of a 1:500 dilution of streptavidin peroxidase in solution A was added to each strip. Wells were incubated at room temperature for 1 hour with gentle rocking and then washed 3 times with PBS/0.05% Tween.

At this point, substrate solution was prepared as follows: 10 ml of methanol in a tightly capped universal, 30 mg of 4-chloro-1-naphthol in a universal and a 15 µl aliquot of H₂O₂ was placed on ice. A volume of 25 ml of NT buffer (10 mM Tris, 0.15 M NaCl, pH 8) was decanted into a 50 ml Falcon tube and placed on the bench until use. When the substrate was required, the methanol was added to the 4-chloro-1-naphthol to make reagent A, and the H₂O₂ was added to the NT buffer to give reagent B. A 5 ml aliquot of reagent A was added to reagent B to produce the active substrate.

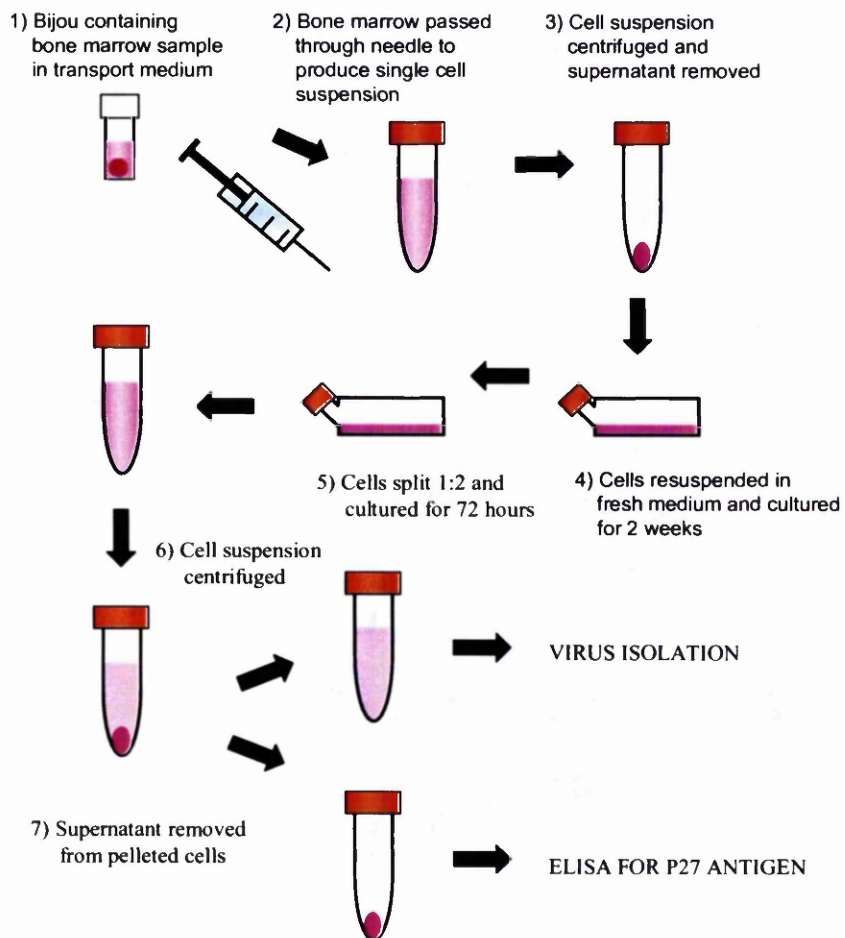
200 µl substrate was added to each strip and the development reaction was stopped by removing the substrate and washing with dH₂O. Antibodies to specific protein antigens were then visualised on the nitrocellulose membrane according to molecular size of protein.

5.2.5.5 Culture of bone marrow cells and viral screening

Bone marrow samples were diluted to 10 ml in bone marrow transport medium and passed first through a 21-gauge needle then a 23-gauge needle in order to produce a single cell suspension. The suspension was centrifuged for 10 minutes at 2000 rpm and the supernatant was removed and stored at -70°C until further use. Cells were resuspended in 10 ml 0.83% ammonium chloride and incubated on ice for 5 minutes to produce erythrocyte lysis. This solution was centrifuged again as above and the supernatant discarded. Pelleted cells were resuspended in 10 ml Alpha Minimal Essential Medium containing 20% FCS, 2 mM L-glutamine, 400 U/ml penicillin/streptomycin and 10⁻⁶ M hydrocortisone succinate (AFC20). Cells were cultured in 25 cm² flasks with 10 ml AFC20 at 2 x 10⁶ cells/ml. Flasks were prepared in duplicate for each sample and they were incubated at 37°C with 5% CO₂.

After 2 weeks of culture, the cells consisted of an adherent confluent monolayer of fibroblasts interspersed with large granulated cells and areas of mature myeloid cells. At this time the bone marrow cells were split 1:2 in AFC20 medium and incubated for a further 72 hours. Cell suspensions were then centrifuged for 10 minutes at 2000 rpm and supernatants were removed and analysed for FeLV by virus isolation as described above. The pelleted cells were used in the p27 ELISA assay detailed earlier to detect the presence of FeLV p27 viral antigen. An overview of the culture of bone marrow and viral screening is shown in figure 5.4.

Figure 5-4 Overview of viral screening of bone marrow samples



5.3 RESULTS

5.3.1 Construction of FeLV DNA vaccine

5.3.1.1 Expression of DNA vaccine plasmids using fixed cell immunofluorescence

In order to demonstrate *in vitro* expression of the FeLV DNA vaccine, pUSE1⁺CMVT(*gag/pol*), pUSE1⁺CMVT(*envA*) and empty pCI-neo vector were transfected into 293T cells and cells were incubated with monoclonal antibodies specific for certain FeLV-A antigens. Antibody specific for p27 antigen detects cells expressing Gag protein and antibody specific for gp70 protein recognises cells synthesising Env protein.

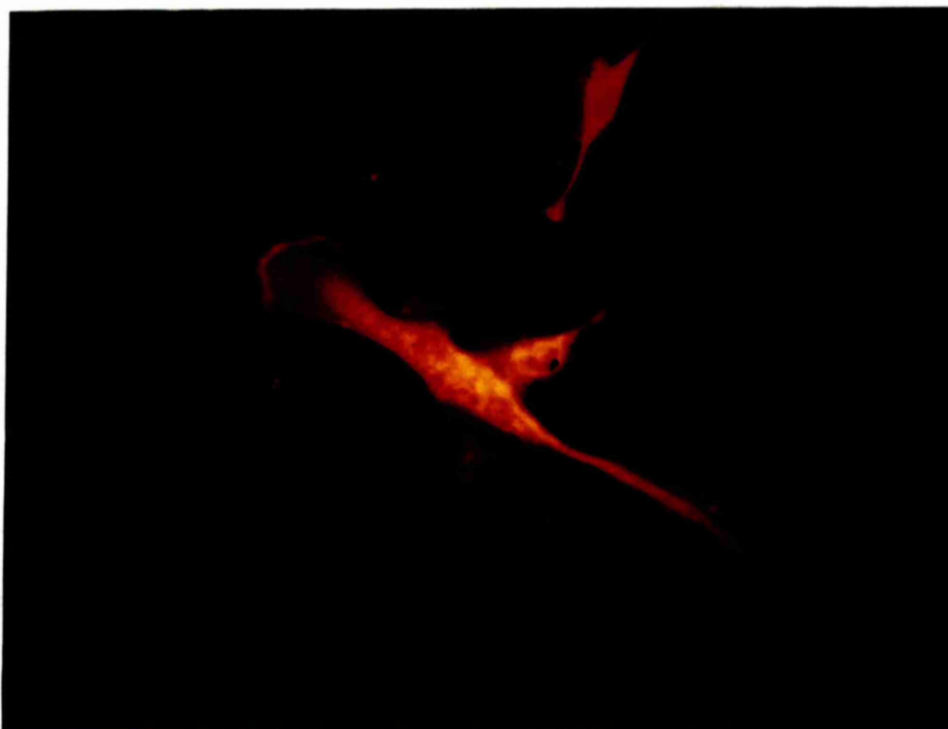
Three cell samples of each transfection were transferred to an 8-well slide system. One well of each transfection was incubated with gp70 antibody, p27 antibody or PBS buffer followed by incubation with IgG FITC conjugate secondary antibody. In addition, positive and negative controls of FeLV-A infected and uninfected FEA cells were prepared alongside. Transfected cells incubated with specific monoclonal antibodies are shown in table 5.5.

Images visualised under UV light are shown in figure 5.5 (a) to (e). Positive and negative controls for antibodies using FeLV-A and FEA cells respectively, are shown in figures (a) and (b). Cells transfected with pUSE1⁺CMVT(*gag/pol*) demonstrated fluorescence when incubated with antibody specific for p27 antigen. No fluorescence could be detected on incubation with gp70 antibody or PBS buffer (d). Similarly, cells transfected with pUSE1⁺CMVT(*envA*) exhibited fluorescence on exposure to gp70 specific antibody but not with antibody specific for p27 or PBS buffer alone (c). To rule out non-specific immunofluorescence, cells transfected with pCI-neo were incubated with the same antibodies. As the images demonstrate, none of these cells exhibited fluorescence (e).

In an earlier experiment, cells were transfected with pCI-neo(*gag/pol*) and pCI-neo(*envA*) and incubated with specific monoclonal antibodies as above. These constructs also displayed immunofluorescence at a similar level to constructs

Figure 5-5 a) Positive and negative control for gp70 monoclonal antibody

FeLV cell line



FEA cell line

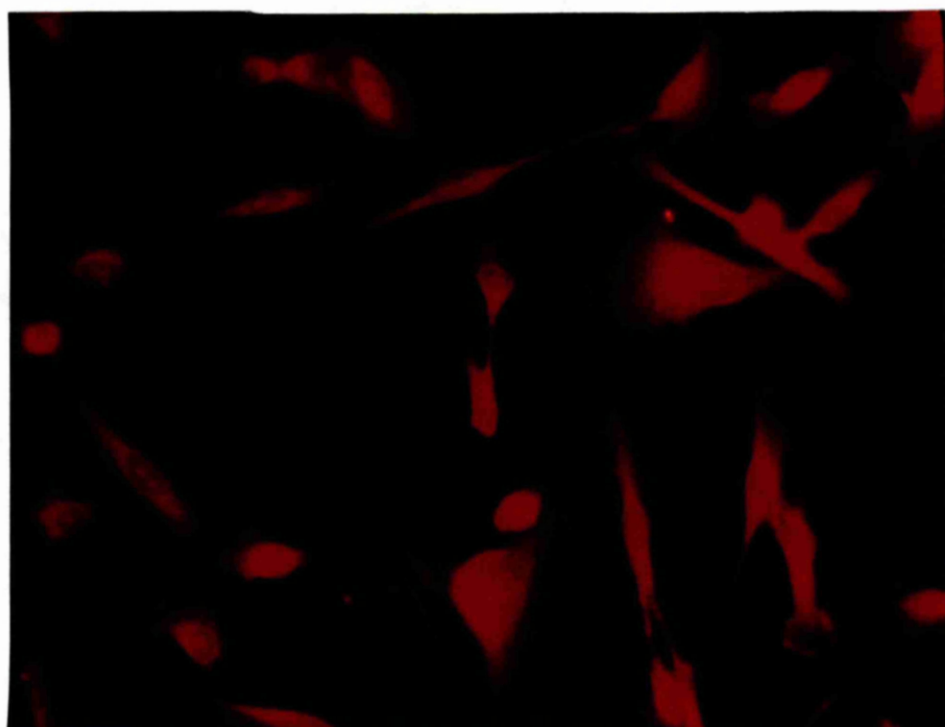


Figure 5.5 b) Positive and negative control for p27 monoclonal antibody

FeLV cell line



FEA cell line

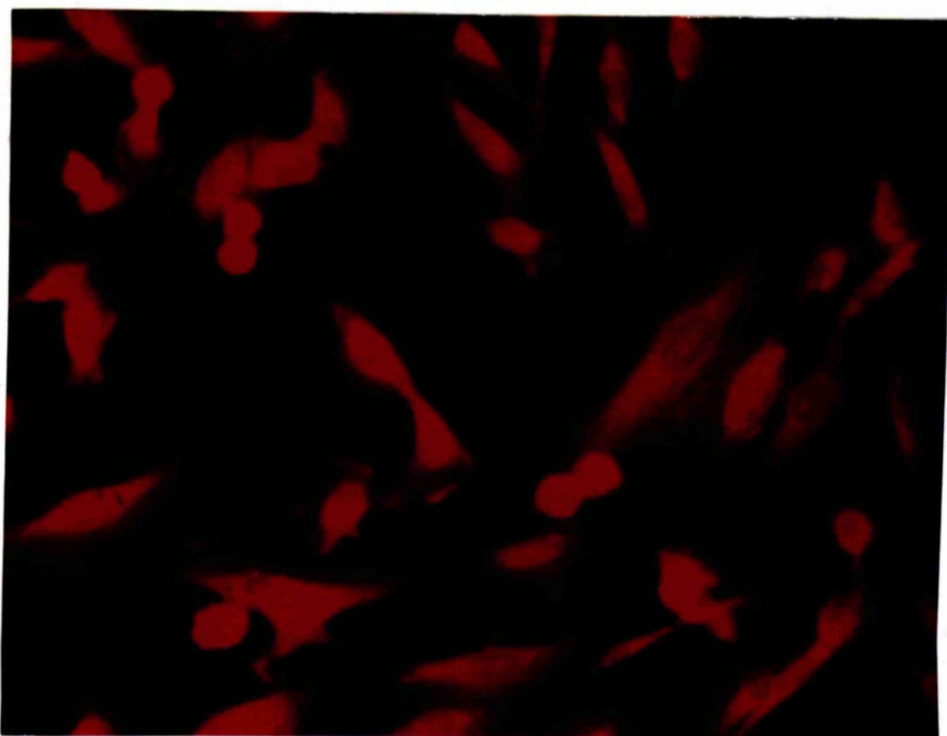
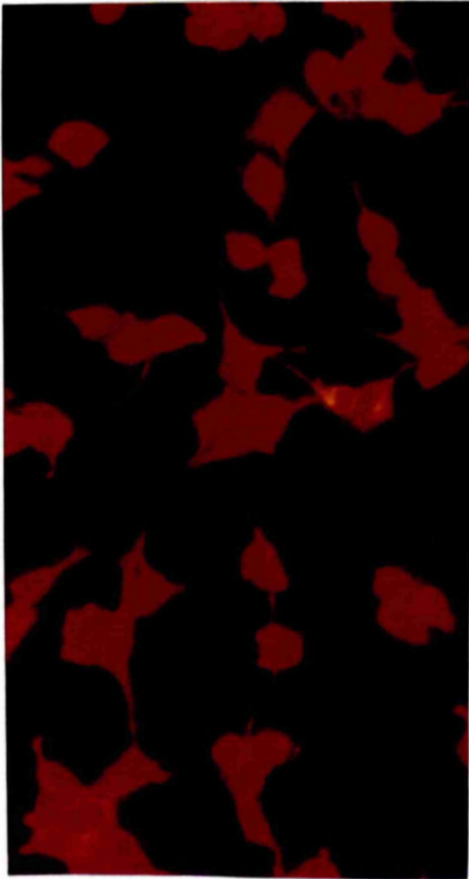


Figure 5.5 c) Immunofluorescence photos of 293T cells transfected with PUSE1⁻ CMVT(*envA*)

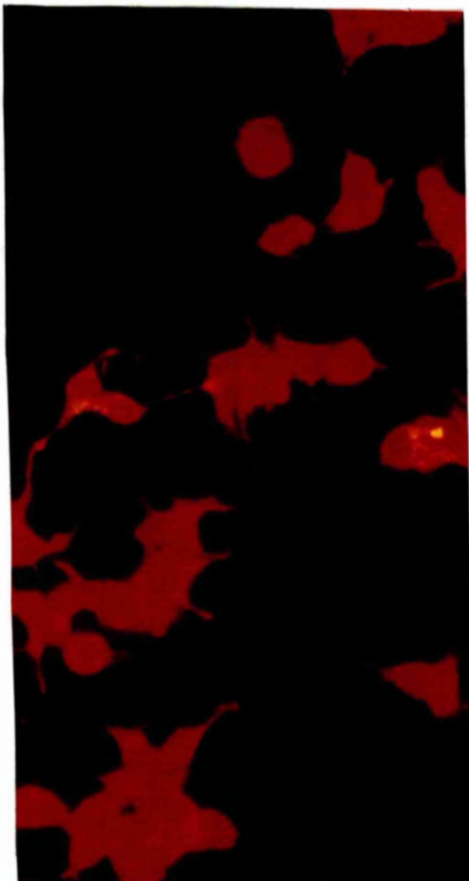
gp70 (SU) monoclonal antibody (x 1)



p27 (CA) monoclonal antibody



gp70 (SU) monoclonal antibody (x 2)



PBS buffer

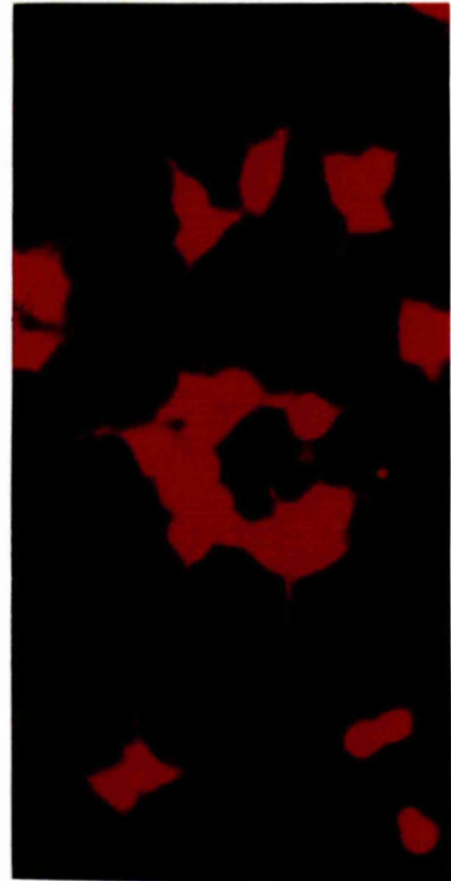
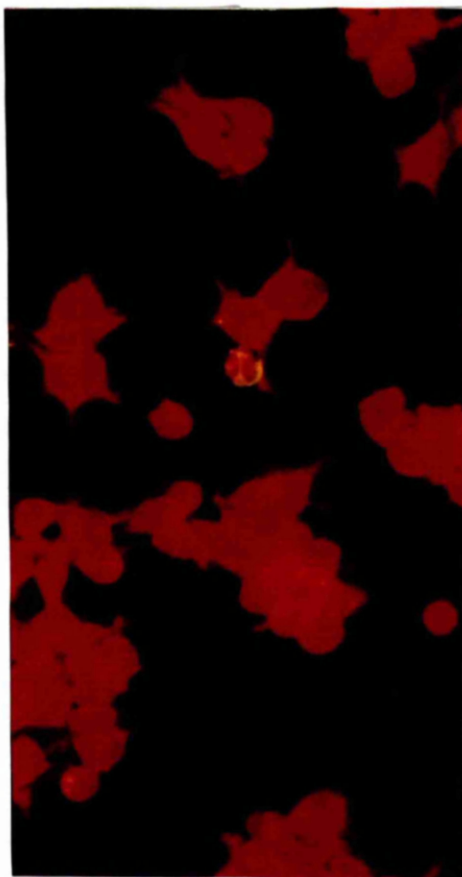
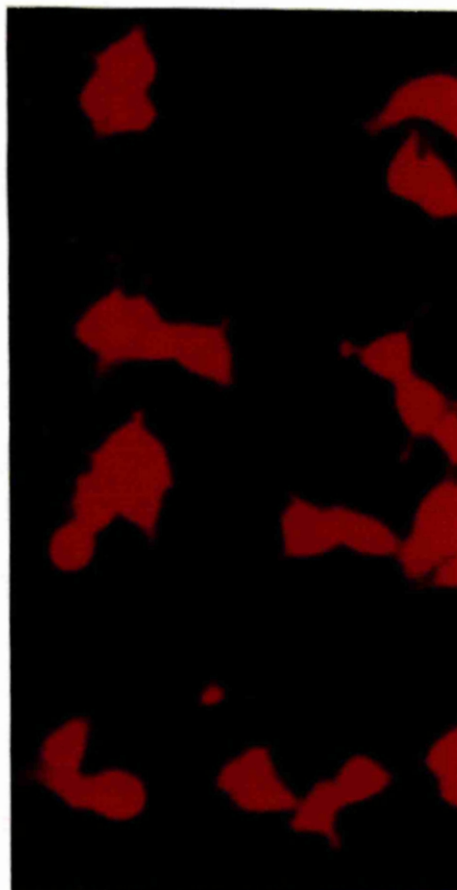


Figure 5.5 d) Immunofluorescence photos of 293T cells transfected with PUSE1⁻ CMVT(*gag/pol*)

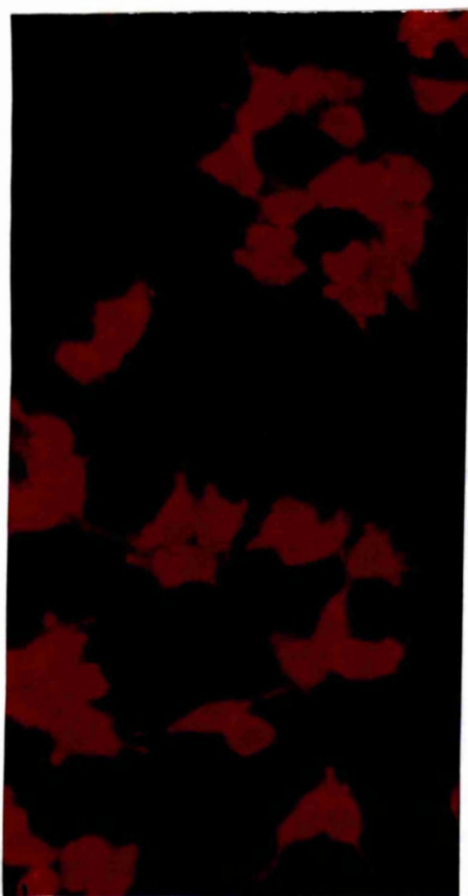
p27 (CA) monoclonal antibody (x 1)



gp70 (SU) monoclonal antibody



p27 (CA) monoclonal antibody (x 2)



PBS buffer

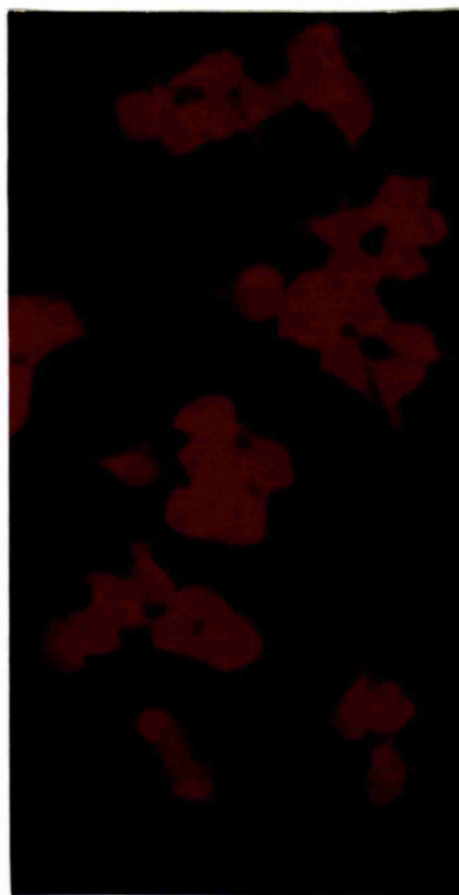
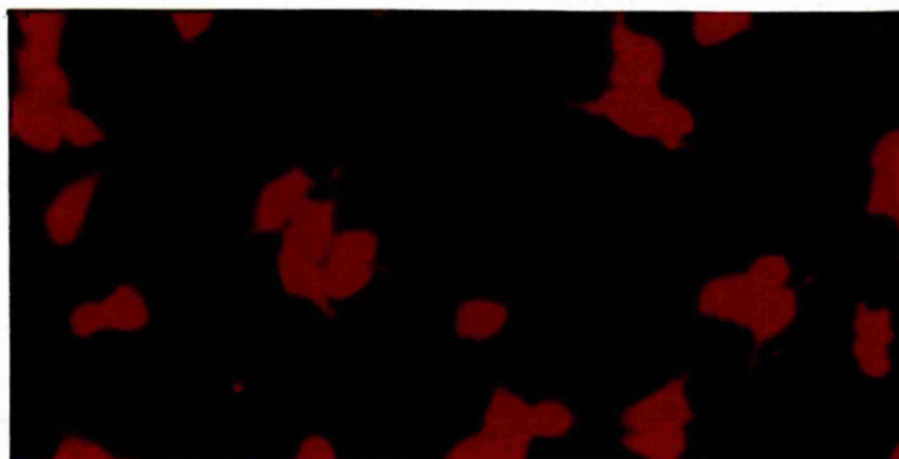


Figure 5.5 e) Negative control immunofluorescence photos of 293T cells transfected with pCI-neo

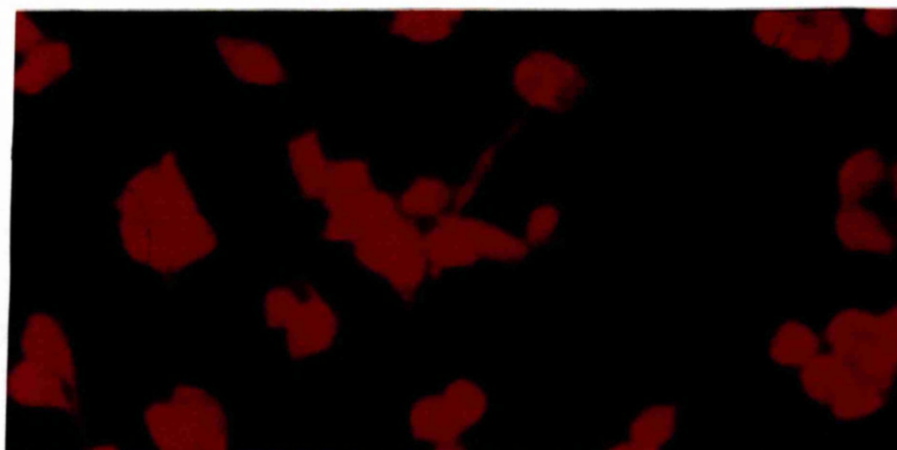
gp70 (SU) monoclonal antibody



p27 (CA) monoclonal antibody



PBS buffer



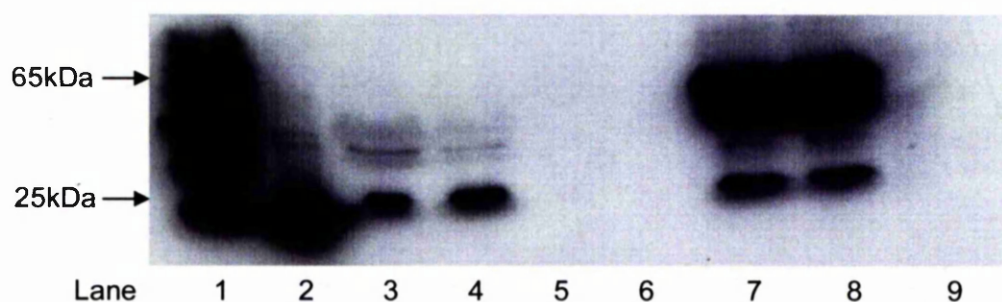
encoded by the pUSE1⁻CMVT expression vector (data not shown), suggesting that the level of antigen expression was unaffected by the plasmid vector used.

5.3.1.2 *In vitro* expression of *gag/pol* constructs using Western blot analysis

The pCI-neo(*gag/pol*) construct was cloned and the level of protein expression from this construct was compared with pUSE1⁻CMVT(*gag/pol*) by Western blot analysis of transfection fluids. Previous *in vitro* work attempting to show protein expression of pUSE1⁻CMVT plasmids had been unsuccessful [Hanlon, 1999]. Therefore the *gag/pol* and *envA* genes were cloned into pCI-neo and compared with pUSE1⁻CMVT construct expression to rule out any aberrant promoter sequence in the pUSE1⁻CMVT plasmids.

A Western blot of 293T transfection products of pUSE1⁻CMVT(*gag/pol*), pCI-neo(*gag/pol*) and pCI-neo vector was prepared.

Figure 5-6 Western blot of 293T transfections of *gag/pol* constructs



Autoradiograph of a 15% SDS-PAGE gel showing supernatants and cell lysates of pUSE1⁻CMVT(*gag/pol*) and pCI-neo (*gag/pol*) 293T cell transfections.

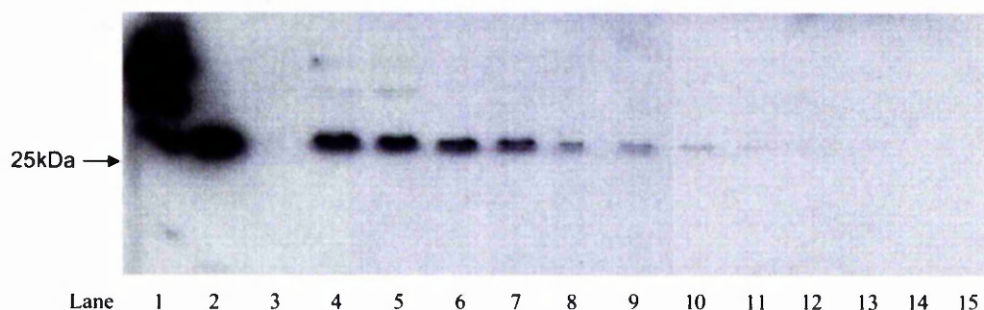
The lane order was as follows: **lane 1:** Novigen protein marker, **lane 2:** F422 cell lysate, **lane 3:** pUSE1⁻CMVT(*gag/pol*) supernatant, **lane 4:** pCI-neo(*gag/pol*) supernatant, **lane 5:** pCI-neo supernatant. **lane 6:** space, **lane 7:** pUSE1⁻CMVT(*gag/pol*) cell lysate, **lane 8:** pCI-neo (*gag/pol*) cell lysate, **lane 9:** pCI-neo cell lysate.

As shown in figure 5.6, the positive control FeLV-infected F422 cell lysate showed a strong band at approximately 27 kDa, indicating strong p27 expression and antibody specificity to this protein. Both pUSE1⁻CMVT(*gag/pol*) and pCI-neo(*gag/pol*) supernatants contained 27 kDa bands which was not evident in the negative control pCI-neo supernatant.

A similar sized protein band was also visualised in both pUSE1⁻CMVT(*gag/pol*) and pCI-neo(*gag/pol*) cell lysates but not in the pCI-neo cell lysate. In addition, two larger bands of around 65 kDa were also present in both cell lysates.

Once p27 protein expression of both constructs was demonstrated, an SDS-PAGE gel was prepared using 1:2 serial dilutions of supernatants of each construct to an end point. This was to compare levels of p27 expression of pUSE1⁻CMVT(*gag/pol*) and pCI-neo(*gag/pol*) in transfected cells.

Figure 5-7 Western blot of serial dilutions of *gag/pol* transfections



Lane 1: Novigen protein marker, **lane 2:** F422 cell lysate, **lane 3:** space, **lanes 4, 6, 8, 10, 12, and 14** are 1:2 dilutions of pUSE1⁻CMVT(*gag/pol*) from neat to 1:32 respectively. **Lanes 5, 7, 9, 11, 13 and 15** are 1:2 dilutions of pCI-neo(*gag/pol*) from neat to 1:32 respectively.

As shown in figure 5.7, similar levels of expression were evident in both supernatants and it was concluded that cloning FeLV *gag/pol* from pUSE1⁻CMVT into pCI-neo had no significant enhancing effect on protein expression of the antigen. Thus, pUSE1⁻CMVT constructs were used in the DNA vaccine trial.

5.3.2 Concentration, purity and endotoxin levels of DNA vaccine batches

High-quality, low endotoxin plasmid DNA was prepared using the Qiagen plasmid Endofree Giga Kit. The concentration and purity of each batch of DNA was estimated by spectrophotometry. The purity of samples was approximated using the ratio of OD readings at 260 nm and 280 nm (OD_{260}/OD_{280}). Pure DNA should have a ratio of approximately 1.8. A lower ratio is suggestive of phenol or protein contamination. Of the batches of DNA used, most ratios were in the region of 1.9 and none had a value lower than 1.81 suggesting that the DNA was of sufficient purity to be used in an *in vivo* experiment. The DNA concentration of batches varied considerably according to the construct being prepared. pUSE1-CMVT(*gag/pol*), pUSE1-CMVT(*envA*), ILRAP-IL-18 and pCI-neo plasmids produced relatively high yields, generally 4-9 $\mu\text{g}/\mu\text{l}$ depending on the volume of PBS in which they were suspended. Flexi-IL-12 produced slightly lower yields requiring more batches of DNA to be prepared. As mentioned previously, batches of DNA were used only if the concentration of endotoxin was measured at less than 50 EU/ mg of DNA. Details of these levels are shown in the table below.

Table 5-8 DNA batches and endotoxin levels used in vaccination trial

Batch	Batch volume (μl)	Concentration DNA ($\mu\text{g}/\mu\text{l}$)	Purity of DNA (OD_{260}/OD_{280})	Endotoxin level (EU/mg)
<i>gag/pol</i> 1	600	9.71	1.90	< 0.51
<i>gag/pol</i> 2	1000	8.00	1.91	9.3
<i>env</i> 4	1000	4.72	1.93	< 1.06
<i>env</i> 5	1000	5.88	1.93	8
ILRAP 1	600	9.44	1.81	< 5.30
ILRAP 2	1000	3.07	1.92	< 1.63
Flexi 1	400	1.52	1.93	< 32.90
Flexi 2	1000	2.17	1.91	< 23.04
Flexi 3	1200	3.47	1.92	36.7
Neo 2	1000	3.53	1.92	< 1.42
Neo 5	1000	7.60	1.92	< 0.66

5.3.3 Statistical analysis of parameters measured during the immunisation trial

Several parameters were measured during this experiment. Virus isolation (VI) and FeLV p27 antigen ELISA were performed on heparinised plasma at various time points throughout the trial. VI produced a positive or negative result depending on the presence of morphological transformation of QN10 cells. A positive value for VI was considered to be definitive for the presence of FeLV viraemia. Animals were classed as persistently viraemic with the development of a positive VI result which was still present at the end of the trial. Transient viraemia was defined as animals that were positive by VI at some point during the experiment but which eventually became VI negative and remained so until the end of the trial.

The p27 ELISA produced an OD result, which was designated as either positive or negative. Empirical data has shown that a positive ELISA OD is a value greater than 1.5 the negative control OD. In addition the ELISA S/P ratio was calculated for each sample using the following equation:

$$\text{ELISA S/P} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}}$$

The purpose of this value is to take into account the positive and negative control values performed during each ELISA which allows for variation occurring between assays. A positive result produces an S/P ratio of greater than 0.1.

The proportions of cats positive and negative for VI and p27 antigen in each trial group were compared pairwise using Fisher's exact test. This test was used to establish whether the protection against viraemia afforded by vaccine groups was statistically significant compared to control groups.

5.3.4 Analysis of data produced from vaccine groups

The two main aims of this vaccination trial were to establish the relative roles of the cytokine adjuvants flexi-IL-12 and ILRAP-IL-18 in enhancing the protection of a

DNA vaccine to FeLV. Also, a previous trial investigating this DNA vaccine used intraperitoneal viral challenge, so the additional aim of this experiment was to expose individuals to a more natural form of FeLV challenge using oronasal inoculation [Hanlon *et al.* 2001]. The vaccine combinations used in each group of cats in the trial are shown below.

Table 5-9 Immunisation groups for current DNA vaccination trial

Group	Cats	Vaccine combination
1	L1 - L6	DNA vaccine + flexi-IL-12 + ILRAP-IL-18
2	L8 - L12	DNA vaccine + flexi-IL-12
3	L13 - L18	DNA vaccine + ILRAP-IL-18
4	L19 - L24	DNA vaccine
5	L25 - L30	pCI-neo vector + flexi-IL-12 + ILRAP-IL-18
6	L31 - L36	PBS

5.3.4.1 Haematological analysis of experimental animals

Haematology samples were taken from all animals before immunisation, on the day of challenge, and 6 and 12 weeks after challenge. These results were generally unremarkable, most values within the approximate normal values for the age of the animal [Jain, 1993]. Some cats displayed slightly high WBC counts, which may have been reflective of mild infection, however this did not seem to be related to viral challenge and the count returned to normal values in the subsequent sample. Five cats showed a neutropenia at six weeks post-challenge, three of which, L8, L22 and L29 were viraemic. In all cases however, these levels had returned to normal 12 weeks after challenge.

5.3.4.2 FeLV p27 antigen, VI and VNAb at time points pre-challenge

Virus was not detected by any of these parameters in animals prior to viral challenge at week 7 (table 5.10). These data show that there had been no exposure of these animals to FeLV prior to experimental challenge. It can therefore be concluded that excluding any antibody response to the DNA vaccine, the subsequent detection of virus or antibody was the result of viral challenge.

Table 5-10 VI, p27 ELISA and VNAb results from start of trial until day of challenge

Cat	PRE IMMUNISATION			48H POST 2ND IMMUNISATION		48H POST 3RD IMMUNISATION		DAY OF CHALLENGE		
	p27	VI	VNAb	p27	VI	p27	VI	p27	VI	VNAb
Group 1 = DNA vaccine + flexi-IL-12 + ILRAP-IL-18										
L1	0.14	-	0	0.15	-	0.13	-	0.13	-	0
L2	0.13	-	0	0.14	-	0.15	-	0.14	-	0
L3	0.14	-	0	0.15	-	0.13	-	0.13	-	0
L4	0.14	-	0	0.14	-	0.14	-	0.15	-	0
L5	0.13	-	0	0.14	-	0.14	-	0.14	-	0
L6	0.13	-	0	0.14	-	0.14	-	0.14	-	0
Group 2 = DNA vaccine + flexi-IL-12										
L8	0.12	-	0	0.14	-	0.13	-	0.14	-	0
L9	0.12	-	0	0.14	-	0.14	-	0.12	-	0
L10	0.12	-	0	0.14	-	0.14	-	0.13	-	0
L11	0.12	-	0	0.15	-	0.15	-	0.14	-	0
L12	0.13	-	0	0.15	-	0.13	-	0.14	-	0
Group 3 = DNA vaccine + ILRAP-IL-18										
L13	0.13	-	0	0.14	-	0.14	-	0.14	-	0
L14	0.12	-	0	0.15	-	0.14	-	0.14	-	0
L15	0.12	-	0	0.14	-	0.14	-	0.14	-	0
L16	0.12	-	0	0.16	-	0.14	-	0.14	-	0
L17	0.12	-	0	0.14	-	0.14	-	0.14	-	0
L18	0.12	-	0	0.15	-	0.13	-	0.14	-	0
Group 4 = DNA vaccine										
L19	0.12	-	0	0.15	-	0.13	-	0.15	-	0
L20	0.13	-	0	0.18	-	0.14	-	0.14	-	0
L21	0.13	-	0	0.15	-	0.14	-	0.15	-	0
L22	0.13	-	0	0.14	-	0.15	-	0.15	-	0
L23	0.12	-	0	0.14	-	0.13	-	0.14	-	0
L24	0.12	-	0	0.14	-	0.14	-	0.13	-	0
Group 5 = 2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18										
L25	0.13	-	0	0.14	-	0.13	-	0.15	-	0
L26	0.13	-	0	0.14	-	0.13	-	0.14	-	0
L27	0.13	-	0	0.14	-	0.14	-	0.15	-	0
L28	0.13	-	0	0.14	-	0.14	-	0.14	-	0
L29	0.13	-	0	0.14	-	0.15	-	0.14	-	0
L30	0.13	-	0	0.14	-	0.13	-	0.14	-	0
Group 6 = PBS buffer										
L31	0.12	-	0	0.15	-	0.14	-	0.15	-	0
L32	0.12	-	0	0.18	-	0.14	-	0.15	-	0
L33	0.13	-	0	0.14	-	0.13	-	0.15	-	0
L34	0.13	-	0	0.14	-	0.13	-	0.15	-	0
L35	0.13	-	0	0.15	-	0.13	-	0.14	-	0
L36	0.13	-	0	0.16	-	0.14	-	0.15	-	0

5.3.4.3 Efficacy of oronasal challenge

One of the main aims of this experiment was to provide an effective viral challenge based on the natural pathway of infection in the field. FeLV is secreted mainly in the saliva and nasal secretions [Hoover *et al.* 1977] and is therefore spread mostly by socialisation of infected animals with susceptible individuals [Hardy *et al.* 1973a]. The method of challenge used here was based on an oronasal viral titration by Harbour *et al.* [2002], which successfully challenged 83% of 15-17 week old cats, where 1×10^6 f.f.u. was administered at day 0, 2, 4 and 8, producing a total dose of 4×10^6 f.f.u. The proportion of latency produced by this dose was not established previously [Harbour *et al.* 2002].

In the current trial, FeLV virus was administered in 1×10^6 f.f.u. doses at days 0, 2, 4 and 8 to cats approximately 5 months of age. Of the negative control cats inoculated with PBS buffer, 4 of 6 cats were positive for p27 antigen at all timepoints between 3 and 15 weeks post-challenge. Three of the cats were VI positive at 6 weeks post-challenge which increased to 4 of 6 cats (66%) at 9 weeks and remained until euthanasia at 15 weeks. In addition, bone marrow culture of the remaining two cats detected latent infection, producing a combined viraemia and latency total to 6 out of 6 cats.

It can be concluded from the data that this method of viral challenge is a successful way of inoculating cats with FeLV, producing a high proportion of viraemic and latently infected cats by simulating a natural route of administration.

Table 5-11 FeLV p27 ELISA results at 3, 6 and 9 weeks post-challenge

CAT	3 WEEKS			6 WEEKS			9 WEEKS		
	ELISA O.D.	ELISA S/P	RESULT	ELISA O.D.	ELISA S/P	RESULT	ELISA O.D.	ELISA S/P	RESULT
Group 1 = DNA vaccine + flexi-IL-12 + ILRAP-IL-18									
L1	0.13	-0.024	-	0.21	0	-	0.17	0.022	-
L2	0.13	-0.024	-	0.18	-0.029	-	0.15	0	-
L3	0.12	-0.048	-	0.17	-0.038	-	0.15	0	-
L4	0.13	-0.024	-	0.18	-0.029	-	0.15	0	-
L5	0.13	-0.024	-	0.17	-0.038	-	0.15	0	-
L6	0.14	0	-	0.18	-0.029	-	0.15	0	-
Group 2 = DNA vaccine + flexi-IL-12									
L8	0.14	0	-	0.53	0.305	+	0.38	0.250	+
L9	0.13	-0.024	-	0.18	-0.029	-	0.15	0	-
L10	0.22	0.190	+	0.70	0.467	+	0.41	0.283	+
L11	0.13	-0.024	-	0.62	0.390	+	0.61	0.500	+
L12	0.13	-0.024	-	0.17	-0.038	-	0.15	0	-
Group 3 = DNA vaccine + ILRAP-IL-18									
L13	0.13	-0.024	-	0.17	-0.038	-	0.15	0	-
L14	0.13	-0.024	-	0.18	-0.029	-	0.15	0	-
L15	0.13	-0.024	-	0.18	-0.029	-	0.15	0	-
L16	0.13	-0.024	-	0.18	-0.029	-	0.15	0	-
L17	0.14	0	-	0.19	-0.019	-	0.16	0.010	-
L18	0.13	-0.024	-	0.18	-0.029	-	0.18	0.030	-
Group 4 = DNA vaccine									
L19	0.44	0.714	+	0.67	0.438	+	0.46	0.310	+
L20	0.13	-0.024	-	0.17	-0.038	-	0.15	0	-
L21	0.13	-0.024	-	0.17	-0.038	-	0.15	0	-
L22	0.13	-0.024	-	0.26	0.048	+	0.27	0.120	+
L23	0.46	0.762	+	0.59	0.362	+	0.71	0.560	+
L24	0.26	0.286	+	0.51	0.286	+	0.48	0.330	+
Group 5 = 2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18									
L25	0.13	-0.024	-	0.18	-0.029	-	0.15	0	-
L26	0.50	0.857	+	0.67	0.438	+	0.37	0.253	+
L27	0.14	0	-	0.18	-0.029	-	0.16	0.010	-
L28	0.27	0.310	+	0.50	0.276	+	0.42	0.313	+
L29	0.44	0.714	+	0.37	0.152	+	0.52	0.434	+
L30	0.14	0	-	0.19	-0.019	-	0.17	0.012	-
Group 6 = PBS buffer									
L31	0.40	0.619	+	0.54	0.314	+	0.33	0.205	+
L32	0.13	-0.024	-	0.19	-0.019	-	0.16	0.010	-
L33	0.34	0.476	+	0.54	0.314	+	0.27	0.133	+
L34	0.43	0.690	+	0.68	0.448	+	0.58	0.506	+
L35	0.58	1.048	+	0.50	0.276	+	0.32	0.193	+
L36	0.15	0.024	-	0.18	-0.029	-	0.15	0	-

Table 5-12 FeLV p27 ELISA results at 12 and 15 weeks post-challenge

CAT	12 WEEKS			15 WEEKS		
	ELISA O.D.	ELISA S/P	RESULT	ELISA O.D.	ELISA S/P	RESULT
Group 1 = DNA vaccine + flexi-IL-12 + ILRAP-IL-18						
L1	0.18	0.018	-	0.20	-0.009	-
L2	0.16	0	-	0.19	-0.019	-
L3	0.16	0	-	0.18	-0.029	-
L4	0.16	0	-	0.17	-0.038	-
L5	0.15	-0.009	-	0.17	-0.038	-
L6	0.15	-0.009	-	0.18	-0.029	-
Group 2 = DNA vaccine + flexi-IL-12						
L8	0.35	0.168	+	0.46	0.238	+
L9	0.15	-0.009	-	0.18	-0.029	-
L10	0.40	0.212	+	0.36	0.143	+
L11	0.56	0.354	+	0.49	0.267	+
L12	0.15	-0.009	-	0.18	-0.029	-
Group 3 = DNA vaccine + ILRAP-IL-18						
L13	0.14	-0.011	-	0.17	-0.038	-
L14	0.15	-0.009	-	0.18	-0.029	-
L15	0.15	-0.009	-	0.18	-0.029	-
L16	0.14	-0.011	-	0.18	-0.029	-
L17	0.15	-0.009	-	0.20	-0.009	-
L18	0.15	-0.009	-	0.18	-0.029	-
Group 4 = DNA vaccine						
L19	0.44	0.330	+	0.79	0.552	+
L20	0.15	-0.009	-	0.18	-0.029	-
L21	0.14	-0.011	-	0.18	-0.029	-
L22	0.32	0.193	+	0.34	0.124	+
L23	0.44	0.330	+	0.61	0.381	+
L24	0.38	0.261	+	0.42	0.200	+
Group 5 = 2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18						
L25	0.15	-0.009	-	0.18	-0.029	-
L26	0.47	0.244	+	0.54	0.314	+
L27	0.15	-0.009	-	0.18	-0.029	-
L28	0.49	0.260	+	0.60	0.371	+
L29	0.73	0.443	+	0.74	0.505	+
L30	0.15	-0.009	-	0.18	-0.029	-
Group 6 = PBS buffer						
L31	0.32	0.130	+	0.51	0.286	+
L32	0.14	-0.011	-	0.18	-0.029	-
L33	0.39	0.183	+	0.59	0.362	+
L34	0.44	0.221	+	0.58	0.352	+
L35	0.62	0.359	+	0.76	0.524	+
L36	0.15	-0.009	-	0.18	-0.029	-

Table 5-13 Number of positive p27 ELISA results in vaccination groups post-challenge

VACCINE GROUP	3 WEEKS	6 WEEKS	9 WEEKS	12 WEEKS	15 WEEKS
vaccine + flexi-IL-12 + ILRAP-IL-18	0/6	0/6	0/6	0/6	0/6
vaccine + flexi-IL-12	1/5	3/5	3/5	3/5	3/5
vaccine + ILRAP-IL-18	0/6	0/6	0/6	0/6	0/6
vaccine	3/6	4/6	4/6	4/6	4/6
2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18	3/6	3/6	3/6	3/6	3/6
PBS	4/6	4/6	4/6	4/6	4/6

Table 5-14 Virus isolation assay results post-challenge

CAT	3 WEEKS	6 WEEKS	9 WEEKS	12 WEEKS	15 WEEKS
Group 1 = DNA vaccine + flexi-IL-12 + ILRAP-IL-18					
L1	-	-	-	-	-
L2	-	-	-	-	-
L3	-	-	-	-	-
L4	-	-	-	-	-
L5	-	-	-	-	-
L6	-	-	-	-	-
Group 2 = DNA vaccine + flexi-IL-12					
L8	-	+	+	+	+
L9	-	-	-	-	-
L10	+	+	+	+	+
L11	-	+	+	+	+
L12	-	-	-	-	-
Group 3 = DNA vaccine + ILRAP-IL-18					
L13	-	-	-	-	-
L14	-	-	-	-	-
L15	-	-	-	-	-
L16	-	-	-	-	-
L17	-	-	-	-	-
L18	-	-	-	-	-
Group 4 = DNA vaccine					
L19	+	+	+	+	+
L20	-	-	-	-	-
L21	-	-	-	-	-
L22	-	+	+	+	+
L23	+	+	+	+	+
L24	-	+	+	+	+
Group 5 = 2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18					
L25	-	-	-	-	-
L26	+	+	+	+	+
L27	-	-	-	-	-
L28	+	+	+	+	+
L29	+	+	+	+	+
L30	-	-	-	-	-
Group 6 = PBS buffer					
L31	+	+	+	+	+
L32	-	-	-	-	-
L33	-	+	+	+	+
L34	+	-	+	+	+
L35	+	+	+	+	+
L36	-	-	-	-	-

Table 5-15 Number of positive VI results in vaccination groups post-challenge

VACCINE GROUP	3 WEEKS	6 WEEKS	9 WEEKS	12 WEEKS	15 WEEKS
vaccine + flexi-IL-12 + ILRAP-IL-18	0/6	0/6	0/6	0/6	0/6
vaccine + flexi-IL-12	1/5	3/5	3/5	3/5	3/5
vaccine + ILRAP-IL-18	0/6	0/6	0/6	0/6	0/6
vaccine	2/6	4/6	4/6	4/6	4/6
2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18	3/6	3/6	3/6	3/6	3/6
PBS	3/6	3/6	4/6	4/6	4/6

Table 5-16 FeLV p27 ELISA and VI results on cultured bone marrow cells

CAT	CULTURED BONE MARROW CELL LYSATE			CULTURED BONE MARROW SUPERNATANT
	ELISA O.D	ELISA S/P	RESULT	VI
Group 1 = DNA vaccine + flexi-IL-12 + ILRAP-IL-18				
L1	0.41	0.365	+	+
L2	0.22	0.108	+	+
L3	0.14	0	-	-
L4	0.14	0	-	-
L5	0.14	0	-	-
L6	0.15	0.014	-	-
Group 2 = DNA vaccine + flexi-IL-12				
L8	1.48	1.444	+	+
L9	0.19	0.011	-	-
L10	1.55	1.522	+	+
L11	1.50	1.467	+	+
L12	0.19	0.011	-	-
Group 3 = DNA vaccine + ILRAP-IL-18				
L13	0.18	0	-	-
L14	0.18	0	-	-
L15	0.19	0.011	-	-
L16	0.18	0	-	-
L17	0.19	0.011	-	-
L18	0.19	0.011	-	+
Group 4 = DNA vaccine				
L19	1.61	1.589	+	+
L20	0.19	0.011	-	+
L21	0.19	0.011	-	-
L22	1.77	1.767	+	+
L23	1.43	1.389	+	+
L24	1.68	1.667	+	+
Group 5 = 2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18				
L25	0.59	0.456	+	+
L26	1.73	1.722	+	+
L27	0.94	0.844	+	+
L28	1.23	1.167	+	+
L29	1.73	1.722	+	+
L30	0.20	0.022	-	-
Group 6 = PBS buffer				
L31	0.81	0.905	+	+
L32	1.03	0.944	+	+
L33	0.68	0.730	+	+
L34	0.75	0.824	+	+
L35	1.66	1.644	+	+
L36	0.23	0.056	-	+

Table 5-17 Number of positive p27 and VI results from bone marrow cell culture

VACCINE GROUP	P27 ELISA OF BONE MARROW C/ L	VI OF BONE MARROW S/N
vaccine + flexi-IL-12 + ILRAP-IL-18	2/6	2/6
vaccine + flexi-IL-12	3/5	3/5
vaccine + ILRAP-IL-18	0/6	1/6
vaccine	4/6	4/6
2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18	5/6	5/6
PBS	5/6	6/6

C/L = cell lysate of cultured bone marrow cells
S/N = supernatant of cultured bone marrow cells

5.3.5 Assessment of DNA vaccines

It should be noted here that the small number of animals in each vaccination group restricted the statistical analysis of the data produced. The efficacy of the vaccine components were compared using p27 ELISA results shown in tables 5.11, 5.12 and 5.13. The first two tables show the OD ELISA result of each animal at sampling points post-challenge. The ELISA S/P ratio is also shown as well as an allocated positive or negative result. Table 5.13 shows the sum of animals positive for p27 in each group at each time point.

Tables 5.14 and 5.15 show VI results between challenge and euthanasia. Table 5.14 shows a positive or negative VI for each animal at each time point. Table 5.15 shows the number of cats VI positive in each group at each time point.

Tables 5.16 and 5.17 show p27 ELISA and VI results performed on cultured bone marrow samples after euthanasia. Table 5.16 displays p27 ELISA OD, ELISA S/P ratio and an allocation of a positive or negative result for ELISA and VI. Table 5.17 shows the number of cats positive for p27 and VI of cultured bone marrow cells in each vaccine group.

5.3.5.1 FeLV DNA vaccination alone does not protect animals from viral challenge

At 3 weeks post-challenge, plasma samples from group 4 (vaccine alone) and group 6 (PBS buffer), demonstrated 2 out of 6 and 3 out of 6 virus positive cats respectively by p27 ELISA and VI. In both cases this increased to 4 cats by 6 weeks post-challenge and remained at this level until the end of the trial. Thus, group 4 demonstrated the same level of protection from challenge as the negative control group 6 animals. It can be concluded from this that the DNA vaccine when administered without adjuvants, did not produce any protective effect from viral challenge.

Neither of the two remaining non-viraemic cats in group 4 (vaccine alone) was shown to harbour latent infection on bone marrow cell culture. However, both of the non-viraemic cats in group 6 (PBS) were latently infected. These results suggest that the vaccine may have stimulated an immune response sufficient to prevent viraemia in

these cats. However, given the small number of animals involved in this experiment, no definite conclusions can be drawn from these data.

To conclude, the levels of infection in group 4 suggest that the DNA vaccine alone is ineffective in providing protection against viraemia, but may stimulate sufficient immunity in some cats to prevent latent infection from developing into viraemia.

5.3.5.2 Cytokine constructs alone are not an effective FeLV vaccine combination

Animals in group 5 were each vaccinated with 200 µg of empty pCI-neo plasmid vector and 100 µg each of flexi-IL-12 and ILRAP-IL-18. This group was therefore injected with the same vaccine combination as group 1 (vaccine + flexi-IL-12 + ILRAP-IL-18) apart from the cDNA encoding *gag/pol* and *env* antigens. This non-specific vector DNA was included to establish whether the actual amount of DNA affected the magnitude of the immune response stimulated. In addition, the vaccine combination of group 5 was used to establish if the cytokine constructs alone induced protection from challenge or whether their efficacy was via an adjuvant effect on the DNA vaccine with which they were administered.

Throughout the 15-week post-challenge period, 3 out of 6 cats were viraemic in group 5 with positive results for both VI and p27. Pairwise comparison with group 6 (PBS) shows that the cytokine constructs alone were not an effective vaccine against challenge even though there might have been a mild degree of protection (3 of 6 cats viraemic compared with 4 of 6 cats). When group 5 was compared with group 1 (vaccine + flexi-IL-12 + ILRAP-IL-18) where there was complete protection from viraemia, a statistical difference of $p=0.091$ was detected. These results suggest that cytokine constructs flexi-IL-12 and ILRAP-IL-18 act by stimulating an adjuvant effect to the DNA vaccine with which they are inoculated. This potent effect is eliminated when the cDNA encoding the antigenic portion of the DNA vaccine is removed. These data also suggest that the actual amount of non-specific DNA used in the vaccine did not significantly affect the immune response elicited.

A similar pattern can be seen in the combination of viraemic and latently infected animals where group 5 showed an infection rate of 5 out of 6 cats. When compared to

group 6 (PBS) and group 1 (vaccine + flexi-IL-12 + ILRAP-IL-18) it is clear that the cytokines alone were not effective at protecting animals from challenge.

5.3.5.3 Flexi-IL-12 does not act as an adjuvant to FeLV DNA vaccination

Unfortunately one cat from group 2 (vaccine + flexi-IL-12) had to be euthanased during the trial leaving only 5 cats in this group. Of these animals, 1 cat became viraemic at 3 weeks post-challenge, which increased to 3 of 5 cats for all subsequent time points until euthanasia. There was no statistical difference between this group and either group 4 (vaccine alone) or group 6 (PBS) at any time point.

At euthanasia, VI and p27 ELISA were performed on cultured bone marrow (BM) cells and cell lysate respectively in order to detect latent infection. As expected, bone marrow of the 3 viraemic cats in group 2 produced virus. The 2 remaining non-viraemic cats, L9 and L12, did not have latent virus suggesting that they were completely protected from infection. The combined total of viraemia and latency calculated for groups 2 (vaccine + flexi-IL-12), 4 (vaccine alone) and 6 (PBS), was 3 of 5 cats, 4 of 6 cats and 6 of 6 cats respectively. There was no statistical difference between group 2 and group 4 or 6 although the numbers suggest that vaccine + flexi-IL-12 may produce a mild degree of protection compared with inoculation of PBS buffer alone.

These results show that in this experiment feline flexi-IL-12 did not act as an adjuvant to the FeLV DNA vaccine and the use of DNA vaccine with flexi-IL-12 did not produce effective protection against viraemia.

5.3.5.4 ILRAP-IL-18 is an effective adjuvant to FeLV DNA vaccination

None of the 6 animals in group 3 (vaccine + ILRAP-IL-18), showed any sign of viraemia at any time point during the immunisation trial. Statistical analysis of VI and p27 results by pairwise comparison of group 3 and group 4 (vaccine alone) at time points 6, 9, 12 and 15 weeks post-challenge gave a value of $p=0.061$. When the p27 and VI results in group 6 (PBS buffer) were compared with group 3 (vaccine + ILRAP-IL-18) using the Fisher's exact test, a statistical difference was found at all timepoints for p27 and all timepoints after 9 weeks post-challenge for V.I ($p=0.061$).

At the end of the trial, although no cats from group 3 (vaccine + ILRAP-IL-18) were positive for p27 ELISA on cultured BM cell lysate, one cat was positive for VI of the cultured BM cell supernatant. As mentioned previously, the combination of viraemia and latency in group 4 (vaccine alone) was 4 of 6 cats. Pairwise comparison of these groups 3 and 4 using Fisher's exact test did not produce a statistically significant result. However the relative number of viraemic cats in each group does suggest that ILRAP-IL-18 functions as an adjuvant to the DNA vaccine. When the combined viraemia and latency of groups 3 and 6 (PBS) were compared using Fisher's exact test, a statistically significant result of $p=0.008$ was produced. This suggests that this vaccine combination of DNA vaccine and ILRAP-IL-18 was effective at stimulating protection against viral challenge when compared with animals inoculated with vaccine buffer alone.

In conclusion, these data suggest that ILRAP-IL-18 plasmid acted as an adjuvant to the DNA vaccine, producing complete protection against viraemia and statistically significant protection against a combination of viraemia and latent FeLV infection.

5.3.5.5 Flexi-IL-12 and ILRAP-IL-18 as adjuvants to FeLV DNA vaccination

Cats in group 1 were inoculated with vaccine, flexi-IL-12 and ILRAP-IL-18 plasmid DNA. During the 15-week post-challenge period, no cats in this group were positive for either p27 or VI. A pairwise comparison of group 1 with group 4 (vaccine alone) and group 6 (PBS buffer) from 6 weeks post-challenge onwards produced a statistical result of $p=0.061$. This implies that the combination of flexi-IL-12 and ILRAP-IL-18 acted as an adjuvant to the DNA vaccine and that the vaccine combination was effective in producing complete protection against persistent viraemia.

At euthanasia, latent infection was detected in 2 of the 6 cats in group 1 on both p27 ELISA and VI. When combined viraemia and latency was compared with group 4 (vaccine alone), the result was not found to be statistically significant although it does suggest that these cytokine constructs have an adjuvant effect on the vaccine. When the combined viraemia and latency of group 1 and group 6 (PBS) were analysed, this produced a statistically significant result of $p=0.061$. These results therefore suggest that the combination of vaccine, flexi-IL-12 and ILRAP-IL-18 does show a degree of efficacy against FeLV infection in this experiment.

5.3.5.6 Clinical scoring system of vaccinated animals

A scoring system was devised to assess the efficacy of each vaccine combination using data produced from plasma and bone marrow samples. Each animal was given a score of either one or zero for seven different parameters depending on whether the result was positive or negative respectively. This produced a maximum score of seven for each animal. The seven parameters were as follows: VI result on plasma at 3, 6, 9, 12 and 15 weeks post-challenge, p27 ELISA result on cultured bone marrow cell lysate and VI result on cultured bone marrow supernatant.

The scores for each vaccination group were combined to give an overall score for animals in that group. This value represented the degree of viraemia and latent infection in each set of individuals. These data are shown in table 5.18 and two bar charts were constructed (figures 5.8 and 5.9). Figure 5.8 is a bar chart comparing the overall clinical score for each vaccine group and figure 5.9 shows the mean clinical score in each group of animals along with the standard deviation for each group. As shown in the figures, the combined total clinical scores and the mean scores in group 6 (PBS) was high and similar scores were produced in group 4 (vaccine alone), group 5 (flexi-IL-12 + ILRAP-IL-18 alone) and group 2 (vaccine + flexi-IL-12). The clinical scores for group 3 (vaccine + ILRAP-IL-18) and group 1 (vaccine + ILRAP-IL-18 + flexi-IL-12) were significantly lower, indicating a lower level of overall infection in these vaccine combinations. Pairwise comparisons of total clinical scores were then made using Fisher's Exact Test (table 5.19). The pattern of statistical difference between groups reflects comparisons of combined viraemia and latency. The vaccine with ILRAP-IL-18 and vaccine with ILRAP-IL-18 and flexi-IL-12 were statistically different to the negative control and the vaccine alone groups ($p < 0.001$), whereas vaccine with flexi-IL-12 alone showed no statistical significance ($p = 0.239$ and $p = 0.485$ respectively). Neither the vaccine alone nor the cytokines alone group demonstrated any statistical significance compared to the negative control group ($p = 0.817$ and $p = 0.495$ respectively), but the vaccine combined with cytokines was statistically significant compared with the cytokines alone ($p < 0.001$). These data provide confirmation that neither DNA vaccine nor cytokines were effective vaccines, whereas vaccine in combination with ILRAP-IL-18 alone or ILRAP-IL-18 and flexi-IL-12 stimulated a high degree of protection against challenge.

Table 5-18 Clinical scores of animals and groups in vaccination trial

	VI POST-CHALLENGE					BONE MARROW			
CAT	3 WEEKS	6 WEEKS	9 WEEKS	12 WEEKS	15 WEEKS	p27 C/L	VI S/N	SCORE	TOTAL
Group 1 = DNA vaccine + flexi-IL-12 + ILRAP-IL-18									
L1	-	-	-	-	-	+	+	2	
L2	-	-	-	-	-	+	+	2	
L3	-	-	-	-	-	-	-	0	
L4	-	-	-	-	-	-	-	0	
L5	-	-	-	-	-	-	-	0	
L6	-	-	-	-	-	-	-	0	4
Group 2 = DNA vaccine + flexi-IL-12									
L8	-	+	+	+	+	+	+	6	
L9	-	-	-	-	-	-	-	0	
L10	+	+	+	+	+	+	+	7	
L11	-	+	+	+	+	+	+	6	
L12	-	-	-	-	-	-	-	0	19
Group 3 = DNA vaccine + ILRAP-IL-18									
L13	-	-	-	-	-	-	-	0	
L14	-	-	-	-	-	-	-	0	
L15	-	-	-	-	-	-	-	0	
L16	-	-	-	-	-	-	-	0	
L17	-	-	-	-	-	-	-	0	
L18	-	-	-	-	-	-	+	1	1
Group 4 = DNA vaccine									
L19	+	+	+	+	+	+	+	7	
L20	-	-	-	-	-	-	+	1	
L21	-	-	-	-	-	-	-	0	
L22	-	+	+	+	+	+	+	6	
L23	+	+	+	+	+	+	+	7	
L24	-	+	+	+	+	+	+	6	27
Group 5 = 2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18									
L25	-	-	-	-	-	+	+	2	
L26	+	+	+	+	+	+	+	7	
L27	-	-	-	-	-	+	+	2	
L28	+	+	+	+	+	+	+	7	
L29	+	+	+	+	+	+	+	7	
L30	-	-	-	-	-	-	-	0	25
Group 6 = PBS buffer									
L31	+	+	+	+	+	+	+	7	
L32	-	-	-	-	-	+	+	2	
L33	-	+	+	+	+	+	+	6	
L34	+	-	+	+	+	+	+	6	
L35	+	+	+	+	+	+	+	7	
L36	-	-	-	-	-	-	+	1	29

C/L = cell lysate of cultured bone marrow cells
S/N = supernatant of cultured bone marrow cells

Table 5-19 Pairwise comparisons of clinical scores using Fisher's Exact Test

Vaccine group	vaccine alone	flexi-IL-12 + ILRAP-IL-18	PBS
vaccine + flexi-IL-12 + ILRAP-IL-18	P<0.001	p<0.001	p<0.001
vaccine + flexi-IL-12	P=0.485	Not applicable	p=0.239
vaccine + ILRAP-IL-18	P<0.001	Not applicable	p<0.001
PBS	P=0.817	P=0.495	Not applicable

Figure 5-8 Barchart showing combined clinical scores for each vaccine group

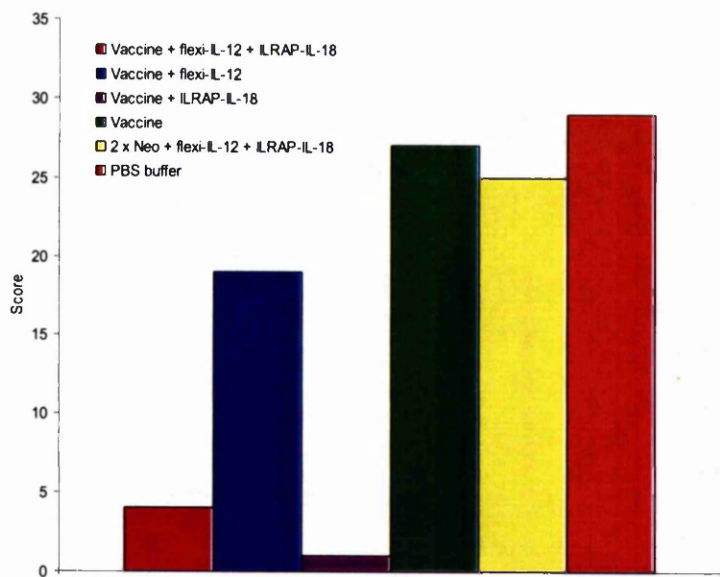
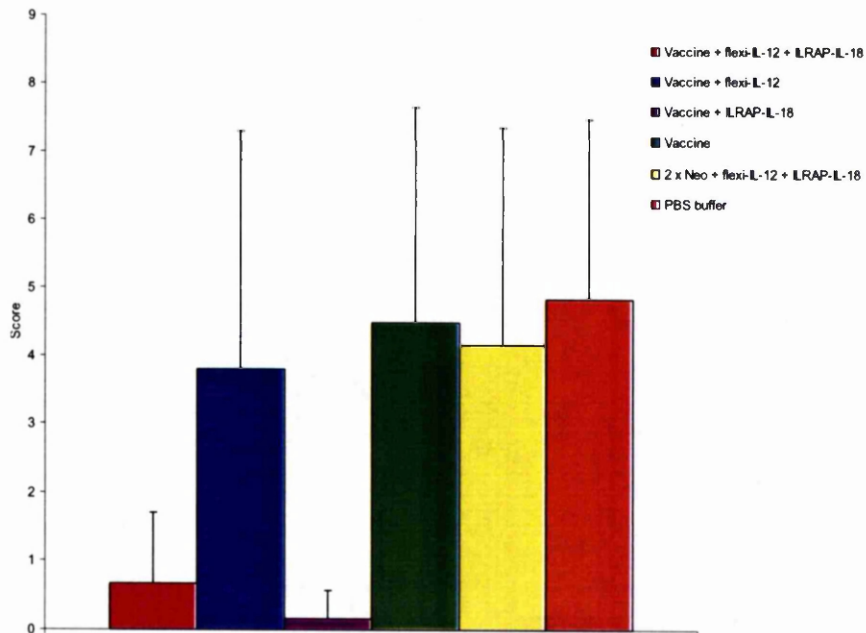


Figure 5-9 Barchart showing mean clinical scores for each vaccine group



5.3.5.7 Virus neutralising and non-neutralising antibodies during trial

VNAb levels were measured before vaccination, on the day of challenge and at the termination of the trial. As detailed previously, there was no detection of antibodies up to and including the day of challenge indicating that the DNA vaccines failed to elicit VNAb prior to challenge. At week 15 post-challenge, the data shows that in general, animals negative for viraemia demonstrated the presence of VNAb and viraemic animals tended to be negative for VNAb (table 5.20). As recorded in table 5.22, only one VI positive cat out of 14 animals (7%) demonstrated VNAb and this was at a low level of 16, whereas 18 of the 21 VI negative animals demonstrated VNAb (86%).

Western blot analysis of complete viral lysate with plasma samples was performed to assess the presence of non-neutralising antibodies at day of challenge and at the termination of the trial. A scanned image of the control and sample strips is shown in figures 5.10 and 5.11 respectively. As shown on the positive control strips, anti-p27 (CA) and anti-gp70 (SU) antibody protein bands can be visualised. Some strips also demonstrate a smaller protein band which represents anti-p15E™ antibody.

Table 5.20 was assembled to assess the presence of NNAb in each animal at the termination of the trial. Also listed here was the viral status and level of VNAb for each individual. A weak positive antibody band was represented by (+) and a strong band by +. Table 5.21 shows the number of cats in each vaccination group positive for each antibody. The percentage of viraemic and non-viraemic animals positive for each antibody is listed in table 5.22.

It can be seen in table 5.21 that the vaccine combination administered to animals did not have any significant effect on the presence or the type of antibody stimulated. The negative control group showed similar numbers of anti-gp70, p27 and p15E antibody positive animals compared to vaccinated animals. All cats inoculated with vaccine and ILRAP-IL-18 had anti-gp70 and p15E antibodies and all were protected from viraemia. This might be expected as VNAb are raised against these antigens and protected cats tend to demonstrate VNAb. However, all animals immunised with

vaccine, ILRAP-IL-18 and flexi-IL-12 were protected from viraemia, but only 3 out of 6 cats had anti-gp70 antibody.

When the proportion of antibody positive viraemic and protected animals was compared (table 5.22), more protected animals had anti-gp70 antibody than viraemic animals (82% and 57% respectively). All protected cats were p15E positive whereas only 64% of viraemic animals demonstrated p15E antibody. However, a higher proportion of viraemic animals expressed anti-p27 antibody than protected individuals (79% and 52%).

Figure 5-10 Positive control western blot strips

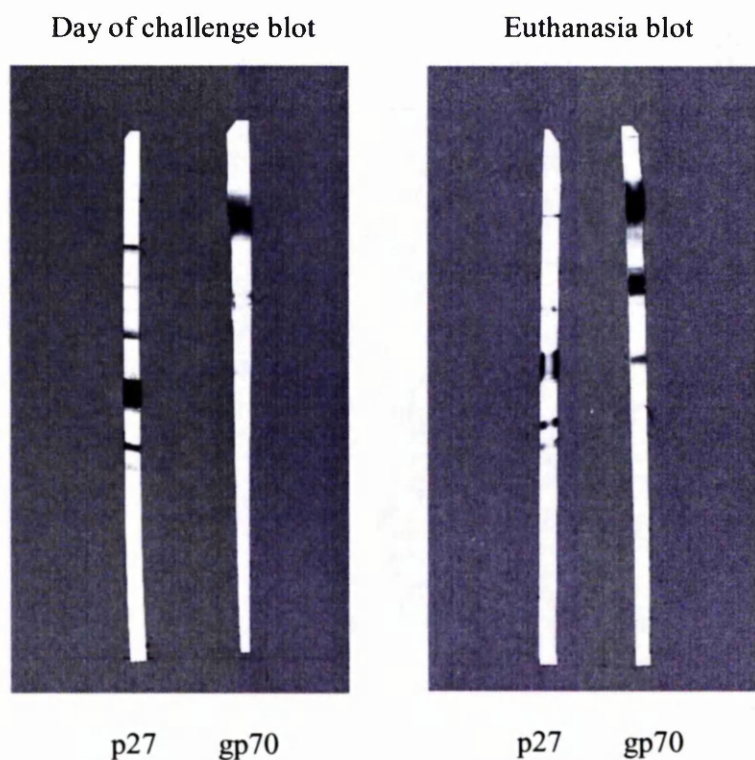
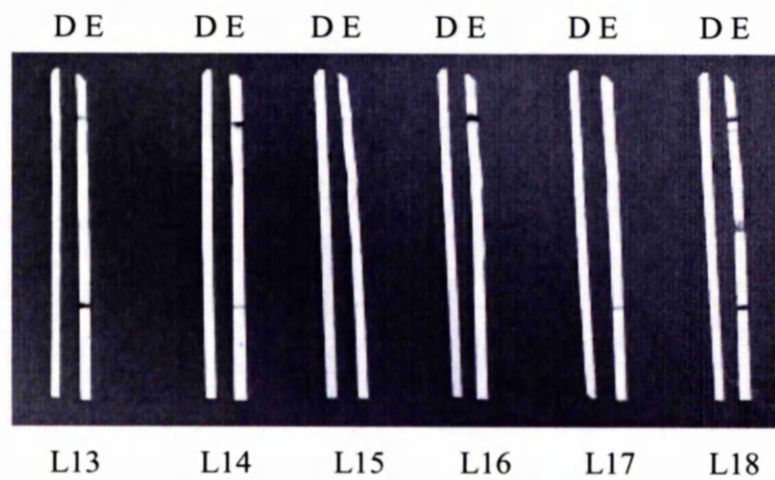
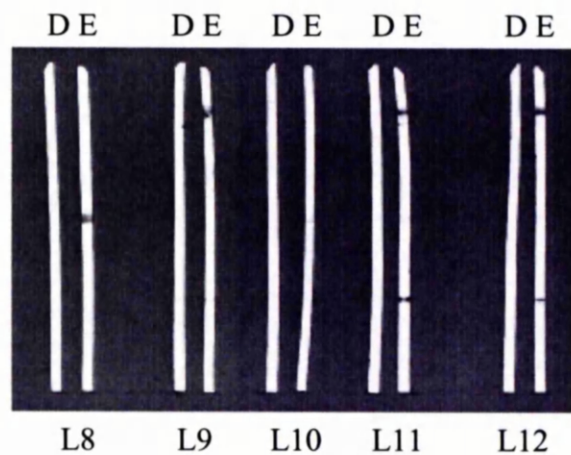
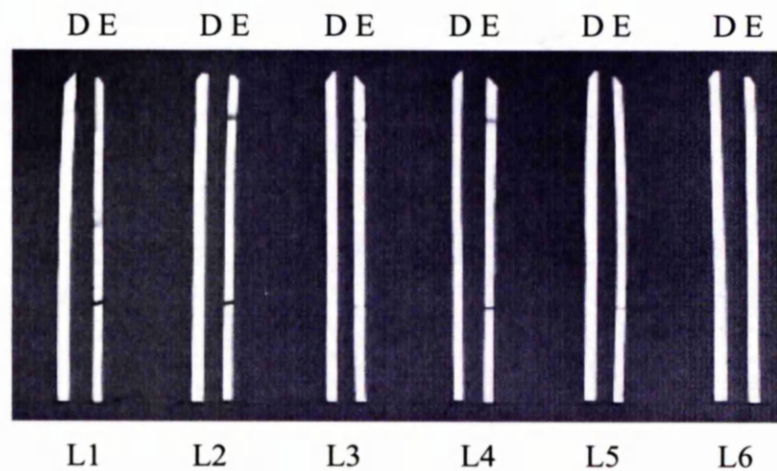
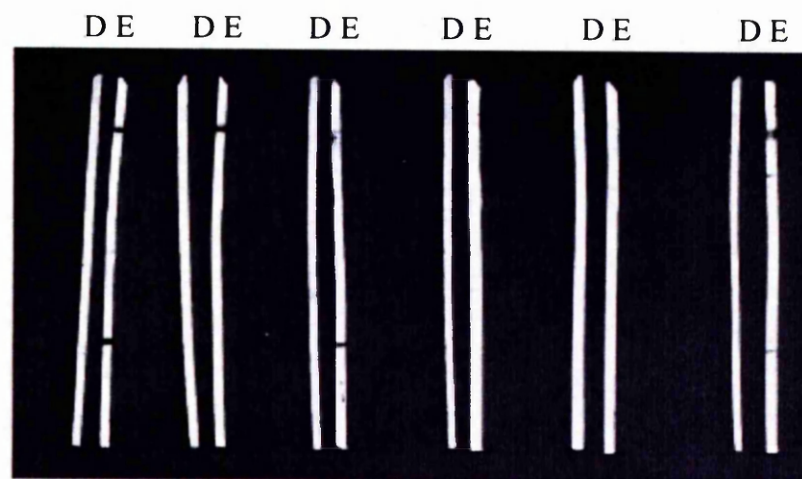


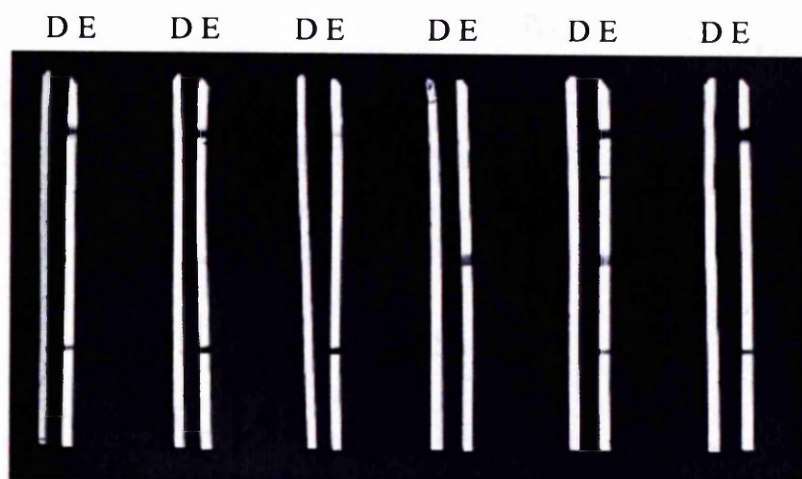
Figure 5-11 Western blot strips of antibodies at day of challenge and euthanasia

D = Day of challenge E = euthanasia

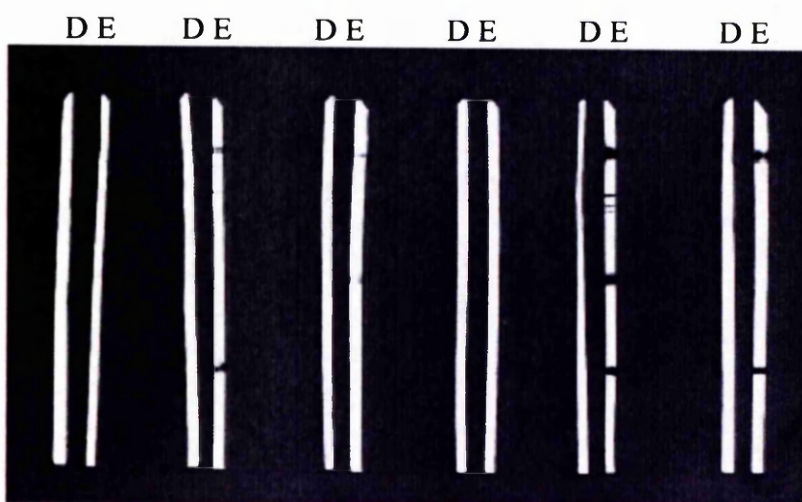




L19 L20 L21 L22 L23 L24



L25 L26 L27 L28 L29 L30



L31 L32 L33 L34 L35 L36

Table 5-20 Viral status and antibodies of animals at 15 weeks post-challenge

CAT	VI	VNAb	anti-gp70 Ab	anti-p27 Ab	anti-p15E Ab
Group 1 = DNA vaccine + flexi-IL-12 + ILRAP-IL-18					
L1	-	16	-	+	+
L2	-	64	+	+	+
L3	-	64	(+)	(+)	+
L4	-	256	+	(+)	+
L5	-	16	-	(+)	+
L6	-	16	-	-	(+)
Group 2 = DNA vaccine + flexi-IL-12					
L8	+	0	-	+	-
L9	-	64	+	-	(+)
L10	+	0	-	(+)	(+)
L11	+	16	+	(+)	+
L12	-	1024	+	(+)	+
Group 3 = DNA vaccine + ILRAP-IL-18					
L13	-	256	+	(+)	+
L14	-	0	+	-	+
L15	-	16	(+)	-	(+)
L16	-	16	+	-	(+)
L17	-	4	(+)	-	+
L18	-	256	+	+	+
Group 4 = DNA vaccine					
L19	+	0	+	+	+
L20	-	0	+	-	(+)
L21	-	64	(+)	(+)	+
L22	+	0	-	-	-
L23	+	0	-	(+)	-
L24	+	0	+	-	+
Group 5 = 2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18					
L25	-	256	+	-	+
L26	+	0	+	(+)	+
L27	-	64	(+)	-	+
L28	+	0	-	+	(+)
L29	+	0	+	+	+
L30	-	0	+	-	+
Group 6 = PBS buffer					
L31	+	0	-	(+)	-
L32	-	1024	+	(+)	+
L33	+	0	+	+	-
L34	+	0	(+)	-	+
L35	+	0	+	+	+
L36	-	16	+	(+)	+

Table 5-21 Number of animals antibody positive in each vaccine group

VACCINE GROUP	VI	VNAb	anti-gp70 Ab	anti-p27 Ab	anti-p15E Ab
vaccine + flexi-IL-12 + ILRAP-IL-18	0/6	6/6	3/6	5/6	6/6
vaccine + flexi-IL-12	3/5	3/5	3/5	4/5	4/5
vaccine + ILRAP-IL-18	0/6	5/6	6/6	2/6	6/6
vaccine	4/6	1/6	4/6	3/6	4/6
2 x pCI-neo + flexi-IL-12 + ILRAP-IL-18	3/6	2/6	5/6	3/6	6/6
PBS	4/6	2/6	5/6	5/6	4/6

Table 5-22 Antibody status of VI positive and negative animals

VI STATUS	% VNAb	% anti-gp70	% anti-p27	% anti-p15E
positive (14/35)	7 (1/14)	57 (8/14)	79 (11/14)	64 (9/14)
negative (21/35)	86 (18/21)	82 (18/21)	52 (11/21)	100 (21/21)

5.4 DISCUSSION

5.4.1 *In vitro* expression of pUSE1⁺CMVT(*gag/pol*) and pUSE1⁺CMVT(*envA*)

As shown in 5.3.1.1, *in vitro* expression of both plasmids comprising the FeLV DNA vaccine was demonstrated using fixed cell immunofluorescence. Only a very small proportion of cells transfected with pUSE1⁺CMVT(*gag/pol*) or pUSE1⁺CMVT(*envA*) was found to express antigen by immunofluorescence. There are several reasons why this may have occurred. Firstly the 293T cells may have demonstrated an inefficient level of transfection. A transfection control of pEGFP-C1 plasmid DNA was carried out alongside the other transfections to monitor the transfection efficiency. The transfection efficiency was found to be at an acceptable level of between 20 and 30% so it is unlikely that the low proportion of cells expressing FeLV proteins was due to transfection efficiency. It is possible that either the plasmid constructs or the protein synthesised from them was toxic, leading to the death of potentially immunoflorescing cells. However, when transfected cells were viewed under the microscope there was no evidence of abnormal cell death. A likely alternative is the efficacy of the monoclonal antibodies used to detect the expression of FeLV proteins. The reason for this is that FEA cells infected with FeLV (FeLV-A cells) also demonstrated low levels of immunofluorescence where all cells were infected with virus and contained large amounts of both p27 and gp70 protein. Use of a higher concentration of monoclonal antibody or a polyclonal antibody may have increased the level of immunofluorescence, but the quantity of antibody available was severely limited preventing investigation of this problem.

5.4.2 *In vitro* expression of *gag/pol* constructs by Western blot analysis

The previous experiment using pUSE1⁺CMVT(*gag/pol*) and pUSE1⁺CMVT(*envA*) *in vivo*, failed to demonstrate expression of the vaccine *in vitro* [Hanlon, 1999]. It was therefore important that the expression of these plasmids was investigated prior to this *in vivo* experiment. If aberrant promoter sequence existed in these constructs, this

could have an effect on the level of protein expression. The *gag/pol* and *envA* genes were therefore cloned into pCI-neo and a comparison of expression of antigens by the different expression vectors was made by Western blot analysis.

The *gag/pol* and *envA* gene were cloned into pCI-neo and parallel transfections of pCI-neo and pUSE1⁺CMVT constructs were performed. Expression of FeLV Env protein from either construct could not be detected by Western blot and therefore comparison of protein expression was not possible. The reason for this may have been that the level of Env protein expressed by the cells was too low to be detected by this system. Alternatively as suggested above, the antibody concentration of the gp70 antibody may have been too low to detect Env protein. As a result, cloning of *envA* into pCI-neo is not discussed in this chapter.

Confirmation of p27 antigen expression by transfections of both *gag/pol* constructs was demonstrated using Western blot analysis. This was initially attempted using streptavidin peroxidase method of detection. Transfection supernatants and lysates were run on a nitrocellulose membrane, cut into strips and exposed to p27 specific monoclonal antibody. This method of protein detection failed to demonstrate p27 antigen expression, but the Western blot analysis system described in 2.2.11 was successful.

A 27kDa protein was evident in both transfection supernatants and cell lysates of pUSE1⁺CMVT(*gag/pol*) and pCI-neo(*gag/pol*). A glycosylated form of FeLV Gag protein is produced by initiation of translation upstream from the normal *gag* initiation codon. This produces Gag protein with an additional signal peptide, allowing protein secretion across the cell membrane [Dickson *et al.* 1985]. However, the p27 present in the transfection supernatant is not the glycosylated protein as the pUSE1⁺CMVT(*gag/pol*) plasmid does not contain sequence upstream of the *gag* initiation codon. In addition, secreted Gag protein is approximately 40kDa in size whereas this protein was 27kDa. This protein may represent the production of recombinant retrovirus particles from *gag/pol* transfected cells. Env proteins have been shown to be unnecessary for virion assembly or budding, as normal appearing virions can be made and released in their absence [Dickson *et al.* 1982]. In addition, a previous study cotransfected pUSE1⁺CMVT(*gag/pol*) and pUSE1⁺CMVT(*envA*) into

293 cells with a LacZ reporter gene. The transfection supernatant was harvested, filtered, and added to FEA cells. Recombinant virus was detected by LacZ expression using a β -galactosidase assay [Hanlon, 1999]. Therefore transfection of cells with pUSE1⁻CMVT(*gag/pol*) alone may allow the production of recombinant retrovirus devoid of Env proteins which would be detected as 27kDa protein using a p27 monoclonal antibody.

In addition to the 27kDa protein band in the cell lysates, two large protein bands of approximately 65kDa were also evident. Gag precursor molecules of approximately 55kDa and 65kDa in size have been found to be synthesised in FeLV infected cells. p55 precursor consists p12, p15 and p27 protein and p65 comprises p10, p12, p15 and p27 protein [Meyer *et al.* 1995; Granoff and Webster, 1999]. The sizes of the bands on the gel are difficult to estimate due to the large amount of protein and low degree of separation, but these bands may represent these Gag precursor molecules.

The amount of p27 protein secreted into the culture fluid of cells transfected by each construct was used as an indicator of the level of protein expressed from each *gag/pol* plasmid construct. One in two serial dilutions of supernatants from neat to 1:32 showed no significant difference in protein expression of each construct. This might be expected as the only difference between pUSE1⁻CMVT(*gag/pol*) and pCI-neo(*gag/pol*) is in the plasmid backbone and pUSE1⁻CMVT expression vector is derived from pCI-neo. It was on this basis that pUSE1⁻CMVT(*gag/pol*) and pUSE1⁻CMVT(*envA*) plasmids were selected as the FeLV DNA vaccine in the immunisation trial. This is because these plasmids had been used successfully in a previous experiment [Hanlon *et al.* 2001], whereas there was no existing *in vivo* data of the efficacy of pCI-neo(*gag/pol*) and pCI-neo(*envA*) as DNA vaccines.

5.4.3 Aims of the vaccination trial

The principal aim of this study was to investigate further the role of the cytokines IL-12 and IL-18 as vaccine adjuvants *in vivo*. Previous data had indicated that a combination of plasmid constructs expressing IL-12 and IL-18 had provided a significant adjuvant effect when coadministered with a DNA vaccine for FeLV

[Hanlon *et al.* 2001]. In that study, the DNA vaccine alone protected only 2 of 6 cats from viraemia whereas the combination of vaccine with both cytokines elicited complete protection. The vaccine with both cytokines demonstrated latent infection in 1 cat compared with 4 cats in the vaccine alone group. The vaccine with IL-12 alone was not found to be effective. These data suggest that either IL-18 was acting alone or that the combination of both cytokines acted synergistically in providing an adjuvant effect to the DNA vaccine [Hanlon *et al.* 2001].

In that study, the lack of a group injected with IL-18 alone in combination with the DNA vaccine prevented the relative role of each cytokine from being established. The current study used the same DNA vaccine with both cytokines alone and in combination in order to establish which component provided the biological effect *in vivo*. As described in Chapters 3 and 4, the cytokine constructs used in the previous trial have each been refined to overcome potential problems of antagonism and secretion. In the case of IL-12, cDNA encoding subunits p40 and p35 were linked by cDNA encoding a synthetic polypeptide linker sequence. This was to prevent the formation of p40 homodimer molecules which theoretically can antagonise the biological effect of the heterodimer. With IL-18, cDNA of the mature molecule was fused to cDNA encoding the signal sequence of the human IL-1 β gene, IL-1 β receptor antagonist protein (ILRAP) to allow secretion of protein from transfected cells. In both cases, *in vitro* expression was demonstrated using Western blot analysis and *in vitro* bioactivity was shown using bioassays specific to each cytokine.

5.4.4 Viral challenge

Several methods of viral challenge have been used in FeLV vaccination experiments. Intraperitoneal inoculation of virus has the advantage of overcoming the resistance to FeLV infection which develops with age [Hoover *et al.* 1976] and is therefore useful in older animals. Natural infection may be simulated by oronasal inoculation of virus, as FeLV is mainly spread by saliva and nasal secretions [Hoover *et al.* 1977]. Field conditions of FeLV disease are most effectively represented experimentally by exposing vaccinates to persistently viraemic cats. It is vital however that the correct proportion of infected cats to vaccinates are used, to guarantee sufficient exposure to virus. Lafrado *et al.* used 5 viraemic cats with 26 vaccinates and produced only 4%

persistent infection [Lafrado *et al.* 1994]. The previous study used intraperitoneal viral challenge and one of the aims of this experiment was to use a more natural form of viral exposure. Therefore to ensure each animal was exposed to a standard amount of virus, oronasal inoculation of virus was used as the route of administration.

As the low number of subjects in each group presented difficulties in statistical analysis, it was crucial that a high proportion of the negative control group became viraemic from challenge. In studies such as this, the dose of virus used for FeLV challenge is difficult to establish, as a standard protocol has never been described. To highlight this problem, table 5.1 shows previously performed FeLV vaccination studies and the protocols used to expose animals to virus after vaccination. These data show clearly that the efficacy of viral challenge varies widely according to dose, age of exposure, type of virus used, route of administration and the way viraemia is defined. This presents great difficulty, not only in selecting an appropriate method of challenge and also comparing the efficacy of different vaccines in these studies.

The dose of virus for the current study was selected on the basis of an experiment performed by Harbour *et al.* [2002], where a dose of 4×10^6 f.f.u. administered in four equal doses on days 0, 2, 4 and 8, produced persistent viraemia in 83% of 15-17 week old kittens. In this study, 4 out of 6 cats became persistently infected after challenge. This was considered a satisfactory level of viraemia and the remaining two cats were found to be latently infected at euthanasia.

In conclusion, the dose of virus administered by the oronasal route was found to be a simple, reliable and robust form of challenge. In future work, a slightly higher viral dose to ensure 100% persistent viraemia could be considered. This method of challenge could also be combined with persistently infected challenge animals to provide an additional source of natural infection to animals in the immunisation trial.

5.4.5 The role of IL-12 as an adjuvant to DNA vaccination to FeLV

It is clear from the results produced from group 2 that IL-12 did not act as an adjuvant to the FeLV vaccine. As mentioned earlier however, the small number of animals in each vaccination group allowed only limited statistical analysis. The fact that one cat in this group also had to be euthanased during the experiment added further difficulty in the analysis of the data produced.

However, of the five animals in group 2, three were persistently viraemic at 15 weeks post-challenge compared to four out of six cats inoculated with vaccine alone (group 4) and PBS buffer (group 6). In terms of viraemia combined with latency, group 2 demonstrated 3 out of 5, group 4 contained 4 out of 6, and group 6 showed all 6 out of 6 cats to be infected. As in the previous study [Hanlon *et al.* 2001], the coadministration of IL-12 plasmid with DNA vaccine appeared to produce no additional protective effect compared to the vaccine alone.

There have been many studies using IL-12 to either increase or modulate the immune response elicited to pathogens and vaccination against pathogen. The role that IL-12 plays in upregulation of the cell mediated immune response has been found to play an important role in defence against infection with pathogens. This stimulation of immunity has led to the use of IL-12 in combination with vaccination to both enhance and alter the nature of the immune response elicited. This has produced successful immune responses in certain viruses, bacteria and protozoa.

However, some studies using IL-12 as an adjuvant have either failed to elicit any effect or have even produced adverse responses *in vivo*. One experiment used IL-12 subunits encoded by separate plasmids in combination with an IL-18 plasmid, as adjuvants to a DNA vaccine to FIV. The same level of protection against FIV challenge was shown with both IL-12 and IL-18 together as with IL-18 alone. The virus-specific CTL responses were measured in each case and the inclusion of IL-12 was found to have no effect on the magnitude of the immune response produced [Dunham *et al.* 2002]. In another study, the immune response to a DNA vaccine for *Leishmania major* was not enhanced by the coadministration of a plasmid encoding murine flexi-IL-12. In contrast, the use of murine flexi-IL-12 alone stimulated a

protective effect in mice [Noormohammadi *et al.* 2001]. The use of an IL-12 plasmid with a DNA vaccine increased the susceptibility of cats to feline infectious peritonitis virus [Glansbeek *et al.* 2002]. Similarly, the use of a single chain IL-12 plasmid was also shown to elicit significant suppression of protective immunity to a DNA vaccine for Japanese encephalitis virus (JEV) [Chen *et al.* 2001].

It has been shown that IL-12 can elicit suppression of both humoral and cell mediated immunity. Further investigation by Chen *et al.* found that IL-12 cDNA produced a dramatic decrease in antibodies specific to JEV. This effect was dose dependent and the suppressive effect in mice was produced after a single inoculation at the initial priming stage of vaccination. The vaccine induced predominantly IgG2a antibodies with IM vaccination and IgG1 following gene gun administration, both of which were abrogated by the coadministration of IL-12 cDNA [Chen *et al.* 2001]. Decreased levels of antibodies were also observed with the coinoculation of IL-12 cDNA with FIPV DNA vaccine [Glansbeek *et al.* 2002].

Cell mediated immunity was also shown to be suppressed in some of these cases. The coinoculation of IL-12 with JEV DNA vaccine decreased the proliferative response of JEV-specific T cells and stimulated lower IFN γ production compared with the vaccine alone [Chen *et al.* 2001]. Similarly, lower levels of peripheral blood leucocytes were observed in kittens post-vaccination when IL-12 was included compared to when FIPV vaccine was given alone [Glansbeek *et al.* 2002].

Some studies have suggested that this immunosuppression may be related to the dose of IL-12 plasmid used. High doses of recombinant murine IL-12 increased the susceptibility of mice to lymphocytic choriomeningitis virus whereas lower doses induced an enhancement of the immune response to the virus [Orange *et al.* 1994]. A recombinant adenovirus expressing IL-12 was shown to potentiate the cell mediated immune response to a hepatitis C vaccine, but at higher doses of IL-12 this adjuvant effect was abrogated. This immunosuppression was accompanied by a dramatic increase in IFN γ , nitric oxide (NO) and apoptosis in the spleen [Lasarte *et al.* 1999]. A similar effect was observed using a recombinant vaccinia virus expressing the *env* gene of HIV-1 and IL-12. When an additional booster of IL-12 was given, the

impairment of immune responses was associated with an IL-12-stimulated increase in NO levels. This suppression was overcome by the use of NO synthesis inhibitors [Gherardi *et al.* 2000]. The stimulation of IFN γ production by IL-12 has been shown to increase the levels of nitric oxide synthesis by macrophages [Waldburger *et al.* 1996]. Nitric oxide inhibits the proliferation of T cells by reducing tyrosine phosphorylation of JAK3 and STAT5 molecules which may help to explain the immunosuppression induced by IL-12 [Bingisser *et al.* 1998].

Increased levels of IL-12 local to the area of immunisation may have also contributed to an impaired immune response. High levels of IL-12 have been associated with immunotoxicities corresponding to inhibition of T cell expansion and CTL activation [Orange *et al.* 1995]. In a study performed by Chen *et al.*, IL-12 plasmid stimulated immunosuppression when coinoculated with JEV vaccine, but not when the vaccine and cytokine were administered simultaneously at different sites [Chen *et al.* 2001]. This suggests that a high level of IL-12 in the local muscle may have had an inhibitory effect on the immune pathway at the site of administration.

The timing of IL-12 inoculation was in certain cases found to be crucial. Immunosuppression was induced when IL-12 was given before or together with JEV vaccine, but not if administered after immunisation [Chen *et al.* 2001]. Further to this, Noormohammadi *et al.* extended their study to show that administration of IL-12 cDNA 4 days after the DNA vaccine to *L. major*, decreased the immunosuppressive effect of IL-12 [Noormohammadi *et al.* 2001]. This was associated with an increase in the level of IFN γ producing T cells.

One of the major modifications to the previous FeLV DNA vaccination study was the use of an IL-12 construct comprising the p35 and p40 subunits encoded by separate expression vectors [Hanlon *et al.* 2001]. The IL-12 construct in this case did not act as an effective adjuvant to the DNA vaccine and it was suggested that this may be due to p40 homodimer formation which may have inhibited the action of this construct [Hanlon, 1999]. It was for this reason that an alternative IL-12 construct was cloned. Flexi-IL-12 prevents the production of p40 homodimer formation by fusion of the two subunits using a synthetic polypeptide linker sequence [Anderson *et al.* 1997] and as

described in Chapter 3, this construct showed *in vitro* protein expression and bioactivity. When used in this immunisation study *in vivo* however, this construct also failed to act as an effective vaccine adjuvant. This suggests that p40 homodimer formation in the previous experiment was not responsible for the lack of *in vivo* efficacy of this construct.

To conclude, these data suggest that various parameters may have inhibited the adjuvant effect of feline flexi-IL-12 in this experiment. The timing of IL-12 administration may have affected both the magnitude and the type of immune response elicited. Also the response to the cytokine construct may have been affected by coadministration, administering the vaccine and flexi-IL-12 at different points may have altered the immune response. In addition, optimisation of the dose of IL-12 may also have enhanced the response, resulting in a higher level of protection.

5.4.6 The role of IL-18 as an adjuvant to DNA vaccination to FeLV

Animals in group 3 were inoculated with FeLV DNA vaccine and ILRAP-IL-18 plasmid construct. Throughout the post-challenge period all cats in group 3 were negative for viraemia, both on VI and p27 antigen ELISA. When the bone marrow of animals was analysed, one cat of six was harbouring virus from group 3 compared to all six animals from group 6 (PBS buffer). This result was statistically significant using the Fisher's exact test, suggesting that DNA vaccine together with ILRAP-IL-18 plasmid provides effective protection against FeLV infection in the bone marrow.

The biological functions of IL-18 make this cytokine an appropriate candidate for use in this *in vivo* situation. Overall, this pleotropic cytokine is an important mediator of cell mediated immunity and Th1 responses and these functions have great potential when applied to DNA vaccination. There are many examples demonstrating the success of IL-18 cDNA as an adjuvant to DNA vaccination. The predominant effect of IL-18 as an adjuvant is the stimulation of a Th1 orientated response. This can be of great value in vaccines against intracellular pathogens such as viruses, bacteria and protozoa. For example, intradermal injection of a plasmid encoding murine IL-18 and its natural signal peptide produced a lower humoral response but a greater cellular

response to *Mycobacterium bovis* Bacillus Calmette-Guerin [Kremer *et al.* 1999]. This construct was also used in a vaccine for *Schistosoma mansoni*, which requires a Th1 response to elicit protection. A DNA vaccine to *S. mansoni* codelivered intradermally with IL-18 plasmid produced significant protection from challenge [Dupré *et al.* 2001]. Similarly IL-18 cDNA was shown to upregulate the cell mediated immune response to DNA vaccines for HIV-1. Coinjection of IL-18 decreased by 2 weeks the time taken to induce a CTL response which corresponded to an enhancement in lymphoproliferative responses specific to the vaccine encoded antigen. In contrast, antibody titres against viral proteins were decreased, demonstrating a Th1 biased immune response [Billaut-Mulot *et al.* 2001a].

The IL-18 plasmid used in this trial consisted of feline mature-IL-18 cDNA fused to a signal sequence for the human IL-1 β gene, IL-1 β receptor antagonist protein (ILRAP). Preliminary work shown in chapter 4 confirmed strong *in vitro* expression and bioactivity of this construct using Western blot analysis and a KG-1 assay respectively. Of particular importance was the evidence of secretion of bioactive protein into the transfection supernatant which was not evident in feline mature IL-18 alone. The *in vivo* data produced in this chapter implies that ILRAP-IL-18 also functions competently in a biological system, producing IL-18 protein capable of stimulating the immune system.

Of interest was the comparison in expression and activity between ILRAP-IL-18 and PsecI-IL-18, a construct used in the previous DNA vaccination trial for FeLV. Western blot analysis using rabbit anti-equine antibody showed minimal levels of PsecI-IL-18 protein expression within the cell and no evidence of protein in the supernatant, which corresponded with background levels of bioactivity on the KG-1 assay. In the previous study, Northern blot analysis demonstrated significant mRNA expression of this construct [Hanlon, 1999] and when this plasmid was used *in vivo*, a significant biological effect was evident [Hanlon *et al.* 2001]. Unfortunately this previous vaccination study did not include a group immunised with vaccine and PsecI-IL-18 alone. However, when the group inoculated with vaccine and IL-12 was compared with the vaccine with the combination of IL-12 PsecI-IL-18, the inclusion of the IL-18 plasmid appeared to induce an additional adjuvant effect.

5.4.7 The role of the combination of IL-12 and IL-18 in FeLV DNA vaccination

Animals in group 1 (vaccine with IL-12 and IL-18) showed no evidence of viraemia at any point post-challenge, detected by p27 ELISA and VI. However, animals injected with the vaccine and IL-18 also demonstrated complete protection against challenge. Therefore it was not possible to establish whether IL-18 was solely responsible for the vaccine adjuvant effect, or whether IL-12 enhanced the immune response even further, as both combinations were completely protective against viraemia. The fact that group 2 (vaccine and IL-12) displayed no statistical difference compared to the control group however, suggests that IL-12 was not an effective adjuvant. Therefore it is more likely that in group 1, IL-18 was an effective adjuvant and IL-12 induced no additional immune response.

Both IL-12 and IL-18 are cytokines which promote the cell mediated immune response by their separate ability to bring about the induction of IFN γ production. Both molecules alone are able to stimulate low levels of IFN γ production, but each can act with costimulatory factors to induce much higher levels. Binding of IL-12 to its cell surface receptor stimulates the signalling pathway of STAT 3 and STAT 4 [Jacobsen *et al.* 1995] which then translocate to the nucleus and bind to promoter regions on the genome, including the IFN γ promoters. IL-12 requires the presence of costimulants to activate the AP-1 binding site of the IFN γ promoter [Barbulescu *et al.* 1998]. IL-18 is also able to augment the production of IFN γ from T cells and is more potent than IL-12 in stimulating its production [Okamura *et al.* 1995]. The signalling pathway of this cytokine uses the adaptor molecule MyD88 which initiates the IRAK pathway, stimulates TRAF6 with the activation of NF-kB and translocation to the nucleus [Robinson *et al.* 1997; Kojima *et al.* 1998; Takeuchi *et al.* 2000]. The production of IFN γ by IL-18 is also greatly enhanced by the use of costimulants [Fantuzzi *et al.* 1998] but unlike IL-12, IL-18 alone produces strong activation of the AP-1 site of the IFN γ promoter [Barbulescu *et al.* 1998].

These two cytokines via their separate pathways have been shown to act together in the synergistic production of IFN γ from both T cells [Micallef *et al.* 1996], and NK

cells [Zhang *et al.* 1997]. Studies have also shown that B cells can also produce IFN γ by stimulation with both IL-12 and IL-18 [Yoshimoto *et al.* 1997]. It has been demonstrated that IL-12-stimulated T and B cells show increased expression of IL-18 receptor, suggesting that IL-12 up-regulates the responsiveness of cells to IL-18 in this way [Yoshimoto *et al.* 1998]. One study used murine mammary carcinoma cells expressing IL-12 and IL-18 by transduction with an IRES IL-12 construct and murine mature-IL-18. Immunisation of these cells induced synergistic protection of 70% mice from carcinoma cells inoculated at a distant site [Coughlin *et al.* 1998].

The results from this study however indicate that the DNA vaccine with ILRAP-IL-18 alone (group 3) was able to provide protection from FeLV, without the requirement of flexi-IL-12. It is possible that the inclusion of IL-12 upregulated the expression of IL-18 receptor on cells, potentiating the activity of IL-18 *in vivo*. From the viraemia data however, it is not possible to establish this, as both ILRAP-IL-18 alone and in combination with flexi-IL-12 induced complete protection from challenge.

5.4.8 Virus neutralising and non-neutralising antibodies produced against FeLV

Until the day of challenge, there was no detection of VNAb in any vaccinated cats. This confirms data produced in the previous trial where no VNAb were detected prior to inoculation of virus [Hanlon *et al.* 2001]. As certain cats in this study were protected against challenge, this implies that an immune response was developed in the initial absence of VNAb.

The active stimulation of VNAb has been shown to have a strong correlation with resistance to infection with FeLV [Hardy *et al.* 1976]. Kittens can also be protected from infection by the passive transfer of VNAb from the mother's milk [Jarrett *et al.* 1977]. This however is not universal: VNAb have been shown not to be essential for FeLV protection and VNAb may be found in cats persistently infected with virus [Charreyre and Pedersen, 1991; Rojko and Hardy, 1994]. In addition, it has been noticed from monitoring VNAb test results that none of the FeLV vaccines currently available in the UK induce consistent VNAb titres immediately after vaccination but

only on recovery from challenge [Jarrett *et al.* 2001]. This suggests that the cellular branch of the immune response may play an important role in protection from infection. Confirmation of this was seen in the previous DNA vaccine experiment where protected cats expressed higher FeLV specific CTLs than viraemic animals [Flynn *et al.* 2000a]. A previous study also showed that FeLV-specific CTLs were detected much earlier than VNAb in FeLV protected cats [Flynn *et al.* 2002].

When the VNAb levels at the termination of the experiment were analysed, it was found that of the 14 viraemic cats, only one, L11, demonstrated a VNAb titre. As mentioned, viraemic cats have been shown to demonstrate VNAb [Charreyre and Pedersen, 1991] and it may have been that the level of antibody produced was insufficient to resist viral infection in this case. Of the 21 protected animals, 18 (86%) demonstrated detectable VNAb titres. This confirms the general correlation between FeLV protection and VNAb levels. However, as stated previously, VNAb are not essential for protection [Charreyre and Pedersen, 1991], and no VNAb was detected in cats L14, L20 and L30, all of which were protected from viraemia. It is possible in these cases that cell mediated immunity may have contributed to protection, but it is not possible to confirm this hypothesis as samples to analyse virus specific CTL levels in animals were not collected.

Detection of non-neutralising antibodies (NNAb) was carried out on plasma samples on the day of challenge and at the termination of the experiment. On the day of challenge there was no detection of antibodies to any of the viral antigens. However, at 15 weeks post-challenge, antibodies to gp70, p27 and p15E antigen could be visualised on Western blot analysis. It has been established that neutralising antibodies to FeLV are raised to the envelope proteins gp70 (SU) and p15E (TM) antigens [Rojko and Olsen, 1984; Rojko and Kociba, 1991]. It is difficult to compare VNAb and NNAb levels of anti-gp70 and anti-p15E due to the different systems used to assay each parameter. It might be expected that an animal with a high VNAb titre would demonstrate detectable gp70 and p15E NNAb levels as it is antibody to these antigens which confer neutralisation of virus. However, the neutralising antibody level may be below the threshold of sensitivity of Western blot analysis. This may be the case in cats L1, L5 and L6, where a low VNAb titre of 16 was displayed with no antibody to gp70 visible by Western blot analysis. However all these cats

demonstrated p15E antibodies which may have induced viral protection. The fact that more protected cats demonstrated gp70 antibody than viraemic cats (82% compared to 57%) might also be expected. However, non-neutralising antibodies raised to gp70 also occur which makes this difficult to assess. Similarly, all protected cats showed detectable p15E antibody compared to 64% of viraemic cats. Again, the antibody detected may be neutralising or non-neutralising depending on the epitope to which the antibody is raised, which makes these proportions difficult to analyse.

Antibodies raised to p27 (CA) antigen were also detected on Western blot analysis. These antibodies are not thought to confer protection against FeLV and these results show that more viraemic cats were positive for p27 than those which were protected (79% compared to 52%). Viraemic animals will have had long term exposure to p27 antigen, not only from viral infection but also from the release of p27 antigen into the blood. Therefore it might be expected that a larger proportion of viraemic cats will show anti-p27 antibodies compared to non-viraemic animals.

5.5 Conclusion

In summary, the work in this chapter shows an *in vivo* study using IL-12 and IL-18 cytokine constructs which *in vitro* demonstrated protein expression, secretion and bioactivity. The constructs were used in combination with a FeLV DNA vaccine in a vaccine trial to investigate the ability of each cytokine alone and together to act as vaccine adjuvants. The ILRAP-IL-18 construct acted as an effective adjuvant, producing complete protection of animals against FeLV viraemia and protection of 5 out of 6 animals against latent viral infection. The flexi-IL-12 construct was not found to act as an effective vaccine adjuvant. When both cytokines were inoculated together, this combination demonstrated complete protection from viraemia and protected 4 out of 6 cats from latent infection. This beneficial cytokine effect was only produced when coinoculated with DNA vaccine, and the vaccine alone stimulated no protective effect. Overall, this is suggestive that ILRAP-IL-18 is initiating the dominant adjuvant effect when used in combination with flexi-IL-12 in this *in vivo* vaccination study.

CHAPTER 6: IMMUNOTHERAPY TRIAL

6 IMMUNOTHERAPY TRIAL

6.1 INTRODUCTION

6.1.1 Immunotherapy of FeLV

As described in the previous chapter, the use of an FeLV DNA vaccine with ILRAP-IL-18 was found to be highly effective at providing immune protection against viral challenge. It was therefore deemed worthwhile to investigate the effect of this vaccine on the viral load of persistently infected cats. Consequently, four persistently viraemic animals from the negative control group of the vaccination trial were inoculated three times at weekly intervals and the viral status of the animals was monitored.

An immunotherapeutic agent for persistently infected animals must have the ability to overcome both the high level of infection and the immunosuppression which is associated with this disease state. Persistently infected animals harbour high viral loads and a large proportion of cells are infected with virus. Furthermore, it is well documented that these individuals rarely develop virus neutralising antibodies [Russell and Jarrett, 1978b; Flynn *et al.* 2002], and that the virus specific CTL response is significantly lower when compared with recovered animals [Flynn *et al.* 2002]. The observation that recovered cats express antibodies to envelope proteins but not Gag proteins, has led to the suggestion that viral immunoevasion may occur. Glycosylated Gag protein released from the cell may induce anergy, inhibiting a CTL response [Jarrett, 1999]. As yet, however, the mechanism behind this immunosuppression of viraemic animals is unknown.

Several studies have adopted an immunotherapeutic approach to the treatment of FeLV infection. One experiment used staphylococcal protein A columns in the removal of IgG and circulating immune complexes from FeLV-infected cats with lymphosarcoma. Of the sixteen cats, complete viral clearance and long-term tumour regression was elicited in nine, and tumour regression without clearance of virus was demonstrated in a further two cats [Snyder *et al.* 1989].

Recent work has investigated the use of immunomodulation therapy in the treatment of clinically ill FeLV-infected cats. Animals were treated with *Staphylococcus* protein A (SPA), oral IFN α or a combination of both therapies. Significantly more pet owners observed improvement in the animals' health with SPA than control animals. However, other than subjective observation, no changes in the clinical parameters of the cats were observed including FeLV status and survival time [McCaw *et al.* 2001]. A study using the combination of IFN α , zidovudine (AZT) and lectin/IL-2 activated lymphocytes was found to clear viral infection in 4 of 9 animals, despite the production of anti-IFN α antibodies. Lymphocytes from this study were taken from putative histocompatible recovered cats that had been exposed to live FeLV virus. This effect was associated with the development of VNAb and 3 of these cats remained virus negative until the end of the experiment, 95 days after treatment. This effect was not seen in animals exposed to activated lymphocytes without IFN α or AZT therapy [Zeidner *et al.* 1995]. Similarly, another study used autologous popliteal lymph node cells to treat persistently infected cats. Lymph node cells were cultured *in vitro* in the presence of IL-2 and were administered intravenously back into the same cat. Clinical improvement was recorded in nine of sixteen animals and four of these became FeLV antigen negative [Blakeslee *et al.* 1998]. Finally the use of a paramunity inducer consisting of inactivated parapox ovis virus is a commonly used drug for FeLV immunotherapy in Germany and other European countries. Several trials however have shown no statistical difference between this drug and a placebo in terms of rate of viraemia in infected cats [Hartman *et al.* 1999].

These studies show that in a proportion of cases, immunosuppression of persistently infected cats can be overcome with the use of immunotherapy. It has been postulated that since the FeLV DNA vaccine with ILRAP-IL-18 was effective in FeLV prophylaxis, it might also have a beneficial effect in viraemic cats through the appropriate presentation of FeLV antigens to the immune system and the development of a cell-mediated immune responses.

6.1.2 Assessment of viral activity

In the previous chapter, the screening of plasma of experimental animals used virus isolation as the definitive way of identifying FeLV viraemia. The vaccination trial only required a positive or negative VI result to ascertain the ability of vaccines to protect against challenge. However, under certain circumstances the degree of retroviral infection may be crucial. This is of vital importance in research into the treatment of HIV, where assessment of viral status is a reflection of the efficacy of the antiretroviral therapy.

The evaluation of viral status requires the measurement of parameters which relate accurately to the extent of infection. This is reviewed in detail by Clementi [2000]. In the case of retroviruses, several quantifiable parameters have been identified based on the dynamic processes of productive infection. The extent of infection may depend on the number of infected target cells, the level of viral production within those cells and the release of infective viral particles. Therefore infection may be measured by the proviral copy number or proportion of infected cells, the level of RNA transcription and the plasma viral load respectively.

6.1.2.1 Proviral copy number

The relationship between PBMC proviral copy number and level of FeLV infection has been studied by Flynn *et al.* [2002]. This work showed that proviral DNA was first isolated in animals 1 week after viral challenge and recovered cats had lower copy numbers of provirus than persistently viraemic animals. Interestingly, the proviral levels during the study were not found to correspond to the level of p27 antigen detected by ELISA [Flynn *et al.* 2002].

6.1.2.2 RNA transcriptional activity

One study measured the genomic RNA copy number of HIV-1 in plasma and the level of HIV-1 transcript copy numbers in peripheral blood CD4⁺ T lymphocytes in untreated patients and those using anti-HIV therapy [Bagnarelli *et al.* 1994]. Patients showed stable RNA levels in the latent clinical phase of infection with dramatically

lower levels in those who had undergone therapy. This suggests that as an indirect parameter, the transcriptional activity of provirus in cells may also correlate with the level of viral infection.

6.1.2.3 Viral loads in plasma

The amount of virus present in the plasma has been shown in various studies to vary in different stages of infection. The quantity of FIV RNA in the plasma was related to the clinical stage, rate of progression and survival time in FIV infected cats [Goto *et al.* 2002]. Another study assessed the level of cytomegalovirus (CMV) in the plasma of individuals with AIDS. Those testing positive for CMV were found to have a 3.4-fold higher chance of developing clinical disease and a 2.5-fold increased rate of death compared to CMV negative individuals. In virus positive cases, a 10-fold increase of CMV in the plasma was found to have a 3.1-fold and 2.2-fold increase in disease rate and mortality respectively [Spector *et al.* 1998].

However conflicting studies have questioned whether these various parameters of viral activity correlate with each other. One study found that the mean RNA HIV copy number per infected cell was constant, irrespective of the viral load in the plasma, although a correlation was found between the proportion of HIV-infected cells in the lymphoid tissue and the plasma viral load [Hockett *et al.* 1999].

6.1.3 Assays used to quantify viral status parameters

Assays used to quantify viral loads are reviewed by Clementi [2000]. These assays are required to be sensitive at both high and low virus levels, robust and reproducible, as well as rapid and easy to perform. In addition, absolute levels of virus must be determined rather than relative values between samples. There are very few assays which serve all of these criteria and most are based on PCR amplification as summarised below.

6.1.3.1 Competitive PCR

Conventional PCR allows specific and sensitive amplification of nucleic acid fragments in samples. However, it does not allow the product to be quantified and as yields increase exponentially with each cycle of amplification, small changes in variables can dramatically influence the amount of product generated.

Competitive PCR is a system that allows reasonable quantification of the RNA or DNA product. This technique employs a competitor DNA fragment that differs from the DNA of interest by including a small intron or a mutated enzyme site. Primers amplify constant ratios of each fragment and these fragments can be quantified [Gilliland *et al.* 1990]. Competitive reverse-transcription PCR was used successfully to monitor the plasma viral loads of cats inoculated with a DNA vaccine against FIV *env* gene [Richardson *et al.* 1997]. However, this system is relatively complex and requires experienced operators to produce reliable results.

6.1.3.2 Branched-DNA *in situ* hybridisation

An alternative to competitive PCR is branched-DNA (bDNA) *in situ* hybridisation. This is a signal amplification system where target DNA or mRNA is hybridised in a microtitre plate well to a series of synthetic branched oligonucleotide probes. This can then be quantified by fluorescent or chromogenic signals via an alkaline phosphatase catalysed reaction. This assay has been shown to be sufficiently sensitive to detect one or two DNA copies per cell. The system was used in human papilloma virus-infected cervical biopsy samples and was found to be rapid, sensitive and highly specific [Kenny *et al.* 2002]. One potential disadvantage of this technique is the requirement for relatively large volumes of sample. For example, one commercial bDNA assay for HIV-1 requires 1 ml of plasma. This problem has been overcome by the development of small-volume-format bDNA which uses 50 or 250 µl of sample with high correlation to the standard test [Yeghiazarian *et al.* 1998].

6.1.3.3 Quantitative real-time PCR (Taqman)

Recently, a PCR method has been developed which measures the amount of product in real time (Taqman). This method uses conventional primers and, in addition, a fluorogenic probe is designed to anneal to the product between the forward and reverse primers on the DNA or cDNA of interest. The probe contains a reporter dye at its 5' end and a quencher at its 3' end. The 5' nuclease action of *Taq* DNA polymerase is then exploited so that cleavage of the probe at its 5' terminus detaches the reporter dye, separating it from the quencher and allowing a fluorescence signal to be emitted (figure 6.1). The 3' end of the probe is blocked to prevent extension of the probe during the PCR reaction. The level of fluorescence provides a direct quantification of the PCR product amplified. This system has been shown to be as sensitive as the other techniques with a wide linear range of amplification. It has the added advantage of being rapid and simple for routine amplification of both RNA and DNA. This is also a real-time system which allows the product to be quantified using the log phase of amplification. The major disadvantage of this system is that initial empirical data that must first be accrued in order to optimise the assay and the fact that sequence variations cannot be quantified.

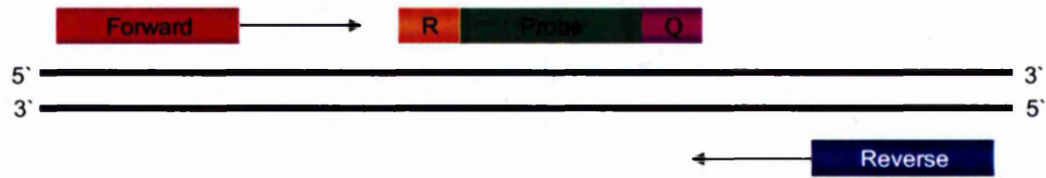
This real-time quantitative PCR system has been used successfully to monitor the level of various retrovirus parameters. One study demonstrated the use of Taqman to detect the level of HIV-1 provirus in PBMCs and lymphoid tissue in individuals undergoing retroviral treatment. The assay was sufficiently sensitive to consistently detect a single proviral sequence and the system demonstrated 100% sensitivity for five copies of provirus [Désiré *et al.* 2001].

This type of assay has also been developed for feline retroviruses. Several studies have successfully used Taqman assays to assess the viral load of FIV in vaccination trials. In one study, primers and a probe were designed from conserved sequence of FIV *gag* gene and used to measure the level of viral RNA in plasma samples. The RNA loads were found to be significantly higher in animals in the AIDS stage of FIV infection than in the asymptomatic stage and high viral loads tended to be associated with poor prognosis [Goto *et al.* 2002]. Quantitative real-time PCR was also used to assess the proviral DNA copy number in buffy coat cells of cats challenged with

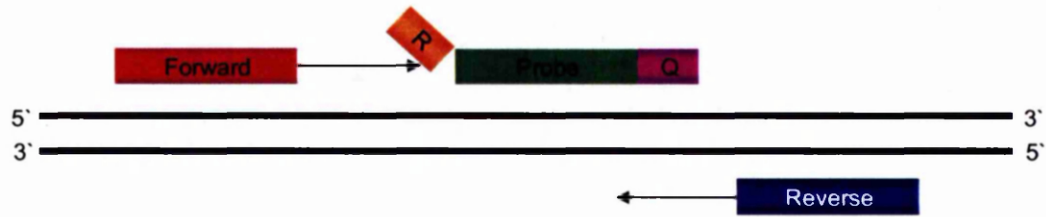
FeLV. Copy numbers were found to be lower in recovered cats than those which were persistently viraemic [Flynn *et al.* 2002]. In addition, one study detected FeLV provirus from experimental infection, using two PCR methods, nested PCR and quantitative real-time PCR [Hofmann-Lehmann *et al.* 2001]. The diagnostic specificity of both methods was found to be 100%. The nested PCR method was found to recognise all three FeLV subgroups, whereas real-time PCR detected FeLV-A/Glasgow and FeLV-B Gardner-Arnstein but not FeLV-C Sarma. Both assays detected between 0.36 and 3.6 copies of DNA and the assays were highly reproducible. There was also a general correlation between the level of p27 antigen in plasma and the mean proviral load in both experimental and naturally infected animals.

Figure 6-1 Quantitative real-time PCR

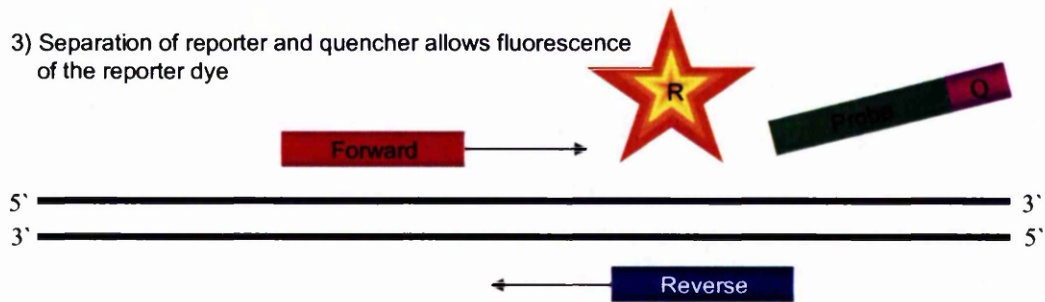
1) Polymerisation of primers and probe to target cDNA



2) Displacement of 5' end of probe allowing reporter detachment



3) Separation of reporter and quencher allows fluorescence of the reporter dye



KEY



Reporter dye



Quencher

6.1.4 Immunotherapy of retroviruses

The use of DNA vaccination in the prevention of infectious disease has been explored extensively. Effective DNA vaccines with adjuvants have been designed for several retroviruses such as HIV-1 [Kim *et al.* 1997], FIV [Hosie *et al.* 1998; Leutenegger *et al.* 2000] and FeLV [Hanlon *et al.* 2001]. However, fewer studies have investigated the use of DNA vaccines in the immunotherapy of retrovirus infection. To date, no experiments have been published on the use of DNA vaccines as immunotherapeutic agents for feline retroviruses. However, several trials have used DNA immunisation in the therapy of HIV-1 infection in both humans and primates.

A DNA vaccination experiment using a plasmid encoding HIV-1 *env* and *rev* was carried out on two HIV-1 infected chimpanzees. One animal was injected with the vaccine and the other with a control plasmid. The vaccinated animal produced an enhancement of humoral responses and a decrease in viral load at week 20 of the study which persisted until the termination of the study. The control chimpanzee was subsequently exposed to DNA vaccine and the same responses were produced [Boyer *et al.* 1997].

Some studies using DNA vaccination in HIV-1 infected humans have demonstrated beneficial responses to DNA vaccination. One experiment used nine patients all of whom were symptom-free [Calorata *et al.* 1998]. After intramuscular injection at days 0, 60 and 180 with plasmids expressing *nef*, *rev* or *tat* genes, all patients showed induction of memory CTL responses and eight had antigen-specific CTL responses. In a third of these patients however, the cellular response was transient and declined after the initial CTL stimulation. Further studies of these nine individuals showed an increase in antigen-specific T cell proliferation responses but no effect on the viral load or the CD4⁺ lymphocyte counts. Patients inoculated with HIV-1 *nef* cDNA induced the highest CTL responses, but all CTL levels in individuals decreased after the initiation of highly active antiretroviral treatment. The increased humoral response elicited by DNA vaccination was also greatest in those injected with *nef* HIV-1 plasmid.

In another study, asymptomatic HIV-1 infected patients were inoculated intramuscularly with plasmids encoding *env* and *rev* of HIV-1 three times at 10 week intervals. DNA vaccination had no effect on either the viral loads, CD4⁺ or CD8⁺ lymphocyte counts in any of these individuals, although some had increases in CTLs against gp160 antigen. Those patients inoculated with larger amounts of DNA (100-300µg) showed increased antibody to gp120. Importantly, the safety and immunogenicity of vaccination was demonstrated and no local or systemic adverse reactions were detected. There was also no trace of anti-DNA antibody in any individual [MacGregor *et al.* 1998].

6.1.5 Aim of this work

The aim of this experiment was to establish the immunotherapeutic value of a DNA vaccine combination, which had previously been effective in protecting animals against FeLV infection.

The experimental trial described in chapter 5 found that the combination of FeLV DNA vaccine with feline ILRAP-IL-18 induced complete protection against viraemia and significant protection against latent proviral infection. Of the control group, four animals became persistently infected. These animals were subsequently exposed to an immunotherapy vaccination schedule using the DNA vaccine with ILRAP-IL-18. Vaccination was carried three times at weekly intervals and blood samples were taken during this procedure and over biweekly intervals until week nine. Plasma was tested for VI, p27 ELISA, VNAb and NNAb. In addition, the proviral load in white blood cells and lymph node cells were established at the end of the trial using a quantitative real-time PCR assay system [Flynn *et al.* 2002].

6.2 MATERIALS AND METHODS

6.2.1 Immunotherapy protocol

6.2.1.1 Experimental animals

Four persistently infected cats from group 6 of the previous immunisation trial were used in this study. These animals had been injected intramuscularly three times at 2 week intervals with PBS buffer, followed by oronasal administration of 4×10^6 f.f.u. of FeLV-A/Glasgow-1 at approximately 5 months of age. This immunotherapy experiment commenced 15 weeks after viral challenge. All procedures in this study were carried out according to Home Office regulations.

6.2.1.2 Preparation of DNA for immunisation

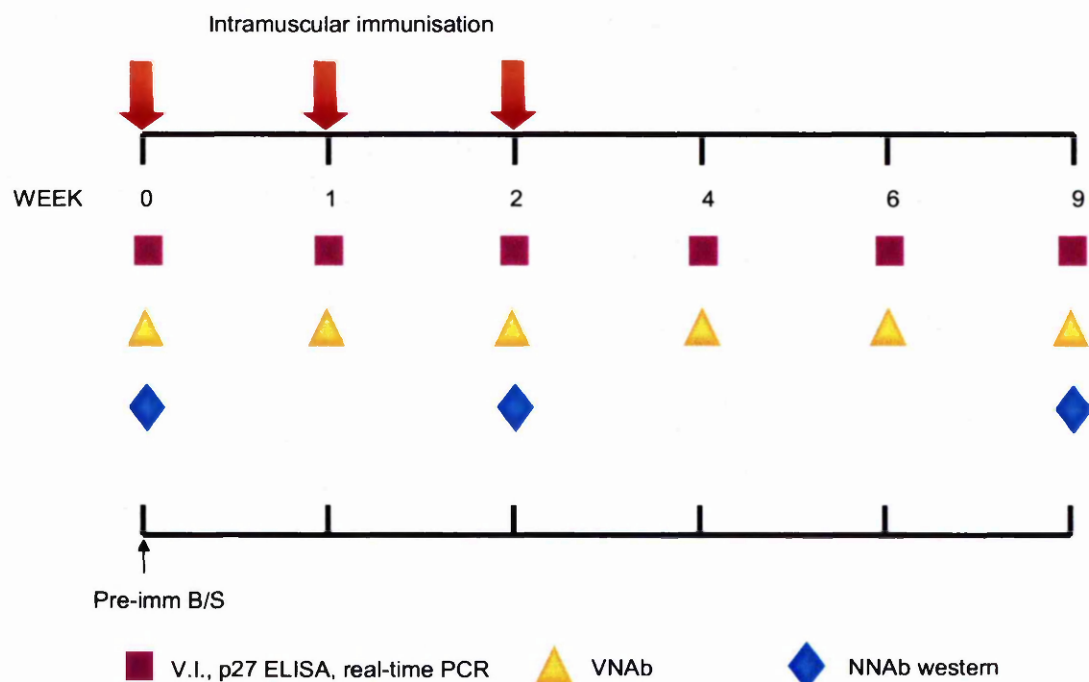
The constructs used for immunotherapy were selected on the basis of the previous vaccination trial. These data showed that the most effective vaccine combination at eliciting protection from viral challenge was the combination of DNA vaccine plasmids and ILRAP-IL-18. Preparation of pUSE1⁺CMVT(*gag/pol*), pUSE1⁺CMVT(*envA*) and ILRAP-IL-18 constructs were carried out as described in 5.2.2, using the Qiagen plasmid Endofree Giga Kit, Qiagen (West Sussex, UK) under the instructions of the manufacturer. Each batch was tested for LAL endotoxin by Biowhittaker Europe, Verviers, Belgium. All DNA used *in vivo* was verified as within the acceptable limits of less than 50 EU/mg of DNA [Schorr *et al.* 1995].

6.2.1.3 Immunisation schedule

The immunisation protocol is shown in figure 6.2. Animals were injected intramuscularly at weeks 0, 1 and 2 with 100 µg each of pUSE1⁺CMVT(*gag/pol*), pUSE1⁺CMVT(*envA*) and ILRAP-IL-18 plasmid constructs. Vaccine DNA was diluted in endotoxin-free PBS so that each inoculation could be administered in a 0.2 ml volume. Vaccination at weeks 0 and 2 was administered into the left quadriceps femoris muscle and at week 1 into the right quadriceps femoris muscle. Animals were

closely monitored after immunisation and no adverse effects to the injection were observed either locally or systemically.

Figure 6-2 Immunotherapy schedule: parameters tested at each timepoint



6.2.1.4 Blood sampling

Animals were manually restrained and blood was taken from either the jugular or cephalic vein. A total of 2 ml of blood was taken at each time point. Cats were sampled at week 0, 1, 2, 4 and week 6. An additional intracardiac blood sample was taken at week 9 from anaesthetised cats immediately before euthanasia.

Of the 2 ml of blood taken, 1 ml was collected into a 2 ml heparin tube and 1 ml into a 2 ml sodium citrate tube. Each 1 ml sample of heparinised blood was centrifuged at 2000 rpm for 5 minutes. The plasma was then removed, dispersed in aliquots and stored at -70°C until further use. Blood collected into sodium citrate tubes was centrifuged for 5 minutes at 2000 rpm and the plasma was removed and stored at -70°C until further use. A small sample of the buffy coat was recovered using a

pipette and transferred to an Eppendorf tube. This was centrifuged at 2000 rpm for 5 minutes, excess plasma was discarded and the pelleted cells were stored at -70°C.

At euthanasia, the same quantities of blood were collected by withdrawal from the heart. In addition, a sample of submandibular (peripheral) lymph node and mesenteric (deep) lymph node were collected separately from each cat into 5 ml bottles each containing 3 ml RPMI 1640 with glutamine and 400 U/ml penicillin/streptomycin. Each sample of lymph node was placed into a Petri dish and 10 ml of fresh medium was added. Cells were extracted from the node by gentle scraping with a sterile scalpel blade. The medium containing the cells was placed in a 15 ml Falcon tube and centrifuged for 5 minutes at 1000 rpm. The supernatant was removed and the pellet was transferred to Eppendorf tubes and stored at -70°C until further use.

6.2.2 Analysis of samples

6.2.2.1 VI, p27 ELISA and antibody analysis

Heparinised plasma was screened for the presence of FeLV by p27 antigen ELISA and VI. Plasma samples were assayed before the start of the trial, during vaccination and at three time points after vaccination. The methods used to perform these assays are described in detail in 5.2.4.6. The VI assay gave a positive or negative result for the presence of virus in the plasma. The ELISA was given a positive or negative result based on whether the OD value was greater than 1.5 times the value of the negative control OD value. The ELISA S/P ratio was calculated as described in 5.3.3.

The VNAb status of each animal at each time point was established, and NNAb were detected pre-immunisation, immediately post-immunisation and at the end of the trial. These assays are explained in detail in 5.3.3.

6.2.2.2 Quantitative real-time PCR

The purification of total DNA from buffy coat and lymphocytes from cats at the six time points was carried out using the QIAamp DNA blood mini kit (Qiagen)

according to the instructions of the manufacturer. Each sample was eluted in 50 µl of Buffer AE and the samples were stored at -20°C until later use.

An initial real-time PCR assay was performed to establish the FeLV proviral DNA copy number per sample reaction. A ribosomal DNA (rDNA) assay was then performed to establish the number of cells in each sample reaction. These two values were combined to produce the number of copies of proviral DNA per cell in each buffy coat or lymphocyte sample.

Assays were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Warrington, UK). Forward and reverse primers and probes were designed using Primer Express software (Applied Biosystems, Warrington, UK). Primers for the detection of FeLV provirus were specific for FeLV-A/Glasgow-1 *env* sequence as shown in figure 6.3 [Stewart *et al.* 1986]. These primers were selected to ensure minimal homology to feline endogenous retrovirus sequence [Pandey *et al.* 1991; McDougall *et al.* 1994] and the primers were synthesised by MWG Biotech and were HPSF purified. The primers were as follows: 5' -GCC CCA AAC GAA TGA AAG C- 3' and 5' -AAT CCG TTT GGG ACC CAT G- 3'. The probe was labelled at the 5' end with the reporter dye FAM (6-carboxyfluorescein), and at the 3' end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine), and was phosphate blocked at the 3' end to prevent *Taq* polymerase extension. The probe sequence was as follows 5'- CCC AAG GTC TGT TGC CCC CAC C -3' (figure 6.3).

The standard for FeLV PCR was plasmid cDNA encoding the FeLV-A *env* gene [Stewart *et al.* 1986]. The copy number of the DNA was quantified by spectrophotometry and from agarose gel electrophoresis as described in 2.2.8. Six standards were used of 10^0 to 10^6 copy numbers in ten-fold dilutions in PCR grade water with 30 µg/ml calf thymus DNA (Invitrogen Life Technologies). A negative control containing no plasmid DNA was also included.

Fifty microlitre reactions were set up containing 10 mM Tris (pH 8.3), 50 mM KCl, 200 nM dATP, dCTP and dGTP, 400 nM dUTP, 2.5 units of *Taq* DNA polymerase, 135 nM of probe (Applied Biosystems), and 300 nM of each primer. A 5 µl volume of

sample DNA and DNA standard was included in each reaction and all samples were assayed in duplicate. PCR conditions comprised an initial denaturation step of 95°C for 2 minutes followed by 40 cycles of amplification of 95°C for 15 seconds and 60°C for 60 seconds.

In order to estimate the cell number in each sample reaction, this assay was repeated using primers specific for rDNA [Klein *et al.* 2000]. The primers were as follows: rDNA343f: 5'- CCA TCG AAC GTC TGCCCT A -3' and rDNA409r: 5'- TCA CCC GTG GTC ACC ATG -3'. The probe for this assay was VIC labelled and was as follows: 5'- CGA TGG TGG TCG CCG TGC CTA -3'. The standards used for this assay consisted of DNA prepared from MYA-1 cells. Quantification of DNA was carried out by spectrophotometry and a series of 4 dilutions was prepared using PCR grade water with 30 µg/ml yeast RNA as a carrier (Roche Diagnostics Ltd., Lewes, UK). The MYA-1 cell number was derived from the quantity of DNA on the assumption that 6 pg DNA represented one cell. A negative control reaction containing no DNA was also included. This assay was carried out under the same conditions as described above and all samples were assayed in duplicate.

Duplicate copy number values from the FeLV proviral DNA assay were used to produce a mean value for each DNA sample and the duplicate rDNA values generated the mean cell number per reaction. These data consequently allowed an estimation of the proviral copies per cell over the course of the immunotherapy trial.

Figure 6-3 FeLV-A *env* of quantitative real-time PCR primers and probe

Key

Red text = Forward primer
Green text = Probe
Blue text = Reverse primer

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1  ATGGAAAGTC CAACGCACCC AAAACCTCT AAAGATAAGA CTCTCTCGTG
51  GAACTTAGCG TTTCTGGTGG GGATCTTATT TACAATAGAC ATAGGAATGG
101 CCAATCCTAG TCCACACCAA ATATATAATG TAACTGGGT AATAACCAAT
151 GTACAAACTA ACACCCAAGC TAACGCCACC TCTATGTTAG GAACCTTAAC
201 CGATGCCTAC CCTACCTAC ATGTTGACTT ATGTGACCTA GTGGGAGACA
251 CCTGGGAACC TATAGTCCTA AACCCAACCA ATGTAAAACA CGGGGCACGT
301 TACTCTCTCT CAAATATGAG ATGTAAACT ACAGATAGAA AAAACAGCA
351 ACAGACATAC CCCTTTTACG TCTGCCCCGG ACATGCCCCC TCGTTGGGGC
401 CAAAGGGAAC ACATTGTGGA GGGGCACAAG ATGGGTTTGT TGCCGCATGG
451 GGATGTGAGA CCACCGGAGA AGCTTGGTGG AAGCCACCT CCTCATGGGA
501 CTATATCACA GTAAAAGAG GGAGTAGTCA GGACAATAGC TGTGAGGGAA
551 AATGCAACCC CCTGGTTTGT CAGTTCACCC AGAAGGGAAG ACAAGCCTCT
601 TGGGACGGAC CTAAGATGTG GGGATTGCGA CTATACCGTA CAGGATATGA
651 CCCTATCGCT TTATTCACGG TGTCCCGGCA GGTATCAACC ATTACGCCGC
701 CTCAGGCAAT GGGACCAAAC CTAGTCTTAC CTGATCAAAA ACCCCCATCC
751 CGACAATCTC AAACAGGGTC CAAAGTGCGC ACCCAGAGGC CCCAACGAA
801 TGAAAGCGCC CCAAGGTCTG TTGCCCCAC CACCATGGGT CCCAACGGA
851 TTGGGACCGG AGATAGGTTA ATAAATTTAG TACAAGGGAC ATACCTAGCC
901 TTAAATGCCA CCGACCCCAA CAAACTAAA GACTGTTGGC TCTGCCTGGT
951 TTCTCGACCA CCCTATTACG AAGGGATTGC AATCTTAGGT AACTACAGCA
1001 ACCAAACAAA CCCCCCCCCA TCCTGCCTAT CTACTCCGCA ACACAACTA
1051 ACTATATCTG AAGTATCAGG GCAAGGAATG TGCATAGGGA CTGTTCTCTAA
1101 AACCCACCAG GCTTTGTGCA ATAAGACACA ACAGGGACAT ACAGGGGCGC
1151 ACTATCTAGC CGCCCCAAC GGCACCTATT GGGCCTGTAA CACTGGACTC
1201 ACCCATGCA TTTCCATGGC GGTGCTCAAT TGGACCTCTG ATTTTGTGT
1251 CTTAATCGAA TTATGGCCCA GAGTGACTTA CCATCAACCC GAATATGTGT
1301 ACACACATTT TGCCAAAGCT GTCAGGTTCC GAAGAGAACC AATATCACTA
1351 ACGGTTGCCC TTATGTTGGG AGGACTTACT GTAGGGGCA TAGCCGCGG
1401 GGTCGGAACA GGGACTAAAG CCCTCCTTGA AACAGCCAG TTCAGACAAC
1451 TACAAATGGC CATGCACACA GACATCCAGG CCCTAGAAGA ATCAATTAGT
1501 GCCTTAGAAA AGTCCCTGAC CTCCCTTCT GAAGTAGTCT TACAAAACAG
1551 ACGGGGCTTA GATATTCTAT TCTTACAAGA GGGAGGGCTC TGTGCCGCAT

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1601 TGAAAGAAGA ATGTTGCTTC TATGCGGATC ACACCGGACT CGTCCGAGAC
 1651 AATATGGCCA AATTAAGAGA AAGACTAAAA CAGCGGCAAC AACTGTTTGA
 1701 CTCCCAACAG GGATGGTTTG AAGGATGGTT CAACAAGTCC CCCTGGTTTA
 1751 CAACCCTAAT TTCCTCCATT ATGGGCCCCCT TACTAATCCT ACTCCTAATT
 1801 CTCCTCTTCG GCCCATGCAT CCTTAACCGA TTAGTACAAT TCGTAAAAGA
 1851 CAGAATATCT GTGGTACAGG CTTTAATTTT AACCCAACAG TACCAACAGA
 1901 TAAAGCAATA CGATCCGGAC CGACCATGA

Table 6-1 FeLV p27 ELISA results before and during immunotherapy

CAT	Week 0			Week 1			Week 2		
	ELISA OD	ELISA S/P	RESULT	ELISA OD	ELISA S/P	RESULT	ELISA OD	ELISA S/P	RESULT
L31	0.51	0.286	+	0.48	0.257	+	0.43	0.210	+
L33	0.59	0.362	+	0.68	0.448	+	0.82	0.581	+
L34	0.58	0.352	+	0.43	0.210	+	0.51	0.286	+
L35	0.76	0.524	+	0.79	0.552	+	0.88	0.638	+

Table 6-2 FeLV p27 ELISA results at three time points after immunotherapy

CAT	Week 4			Week 6			Week 9		
	ELISA OD	ELISA S/P	RESULT	ELISA OD	ELISA S/P	RESULT	ELISA OD	ELISA S/P	RESULT
L31	0.46	0.238	+	0.51	0.286	+	0.39	0.256	+
L33	0.86	0.619	+	0.79	0.552	+	0.69	0.605	+
L34	0.53	0.305	+	0.49	0.267	+	0.58	0.477	+
L35	0.73	0.495	+	0.64	0.410	+	0.54	0.430	+

Table 6-3 Virus isolation results of immunotherapy trial

CAT	Week 0	Week 1	Week 2	Week 4	Week 6	Week 9
L31	+	+	+	+	+	+
L33	+	+	+	+	+	+
L34	+	+	+	+	+	+
L35	+	+	+	+	+	+

Table 6-4 Non-neutralising antibody levels during immunotherapy trial

CAT	Week 0	Week 2	Week 9
L31	No antibodies	No antibodies	No antibodies
L33	gp70 (+), p27 (+), p15E (+)	gp70 (+), p27 (+), p15E (+)	gp70 +, p27 (+), p15E (+)
L34	p15E +	gp70 (+), p15E +	p15E +
L35	gp70 +, p27 +, p15E +	gp70 +, p27 +, p15E +	gp70 +, p27 +, p15E +

Table 6-5 Quantitative real-time PCR results of immunotherapy trial

	Week 0	Week 1	Week 2	Week 4	Week 6	Week 9	SMLN	MLN
L31								
Mean provirus copy number	1060811	517181	1020237	1675689	1815755	1013534	85409	49428
Mean cell number	96652	76624	658273	580406	116865	510055	140435	115332
Copy number/cell	10.976	6.750	1.550	2.887	15.537	1.987	0.608	0.429
L33								
Mean provirus copy number	257983	269579	427709	318796	387816	417523	41089	47244
Mean cell number	1564872	185042	706973	832651	393276	1557837	185786	273245
Copy number/cell	0.165	1.457	0.605	0.383	0.986	0.268	0.221	0.173
L34								
Mean provirus copy number	389761	83734	104208	54664	85247	229358	33570	16433
Mean cell number	3813909	140965	412030	138312	27228	476357	360160	111657
Copy number/cell	0.102	0.594	0.253	0.395	3.131	0.481	0.093	0.147
L35								
Mean provirus copy number	35840	16184	16409	11010	22873	80091	2364	39023
Mean cell number	416118	23561	15761	56093	85339	204127	40823	114728
Copy number/cell	0.086	0.687	1.041	0.196	0.268	0.392	0.058	0.340

SMLN = submandibular lymph node

MLN = mesenteric lymph node

6.3 RESULTS

6.3.1 The effect of immunotherapy on FeLV viraemia

Heparinised plasma was used to establish the viral status and antibody response of animals during the study. VI and p27 antigen ELISA assays were performed on plasma samples and each cat at all time points was found to contain p27 antigen (table 6.1 and 6.2) and infectious virus (table 6.3).

6.3.2 The effect of immunotherapy on FeLV proviral DNA

Quantitative real-time PCR was performed on duplicate DNA samples to estimate the mean FeLV proviral copy number per cell from buffy coat samples collected over all time points and lymph nodes collected at euthanasia. For each assay a standard curve was constructed using the standard reactions and the FeLV or rDNA copy number was calculated by interpolation of the C_t value of the samples to the standard curve (figure 6.5). Primers designed to amplify FeLV-A *env* were used to approximate the FeLV copy number per reaction and primers for rDNA allowed the number of cells per reaction to be estimated. This allowed an estimation of the mean number of proviral copies per cell sample (table 6.5). A graph was then constructed of \log_{10} of proviral copy number for each animal for the duration of the trial (figure 6.6).

The graph clearly shows that throughout the immunotherapy experiment, the proviral copy number per cell fluctuated randomly around a low general level. There was no overall increase or decrease in copy number in any animal over the study period. When the mean copy number per cell was calculated for each animal using the six buffy coat samples they were as follows L31=6.61, L33=0.644, L35=0.826 and L35=0.445 copies/cell.

There appeared to be little correlation between the copy numbers found in lymph node cells and the buffy coat samples, other than L31, which overall had much larger values than the other animals for all samples.

In order to compare proviral copy number per cell and p27 ELISA values, a graph was constructed of FeLV p27 ELISA S/P ratios at time points for each animal (figure 6.7) and a correlation curve of ELISA S/P against proviral copy number was constructed using SigmaPlot 2001 (Windows version 7.0, SPSS Inc.). Proviral copy number was plotted on a log scale. The R value of points on the correlation curve was found to be $R=0.433$ which shows that there is no correlation between these values (figure 6.8). This suggests that the OD value produced from p27 ELISA does not necessarily reflect the degree of proviral infection of cells.

6.3.3 The effect of immunotherapy on anti-FeLV antibodies

The levels of VNAb and NNAb were analysed at various intervals during the study. VNAb was not detected in animals at any time point during the experiment. Detection of NNAb carried out at weeks 0, 2 and 9 showed no significant change in the level of NNAb (figure 6.4 and table 6.4). Of the animals, L31 showed no NNAb at any point, whereas L33 demonstrated low levels of anti-gp70, p27 and p15E antibodies. The anti-gp70 antibody band for L33 became slightly stronger at each time point during the trial. L34 also displayed low levels of anti-p15E and anti-gp70 antibody was detectable at week 2. L35 demonstrated all antibodies at all times with no consistent change in the strength of antibody bands at the various time points. An interesting observation was that there tended to be an inverse relationship between proviral DNA copy numbers and the level of non-neutralising antibodies expressed by these animals.

Figure 6-4 Western blot strips of non-neutralising antibodies

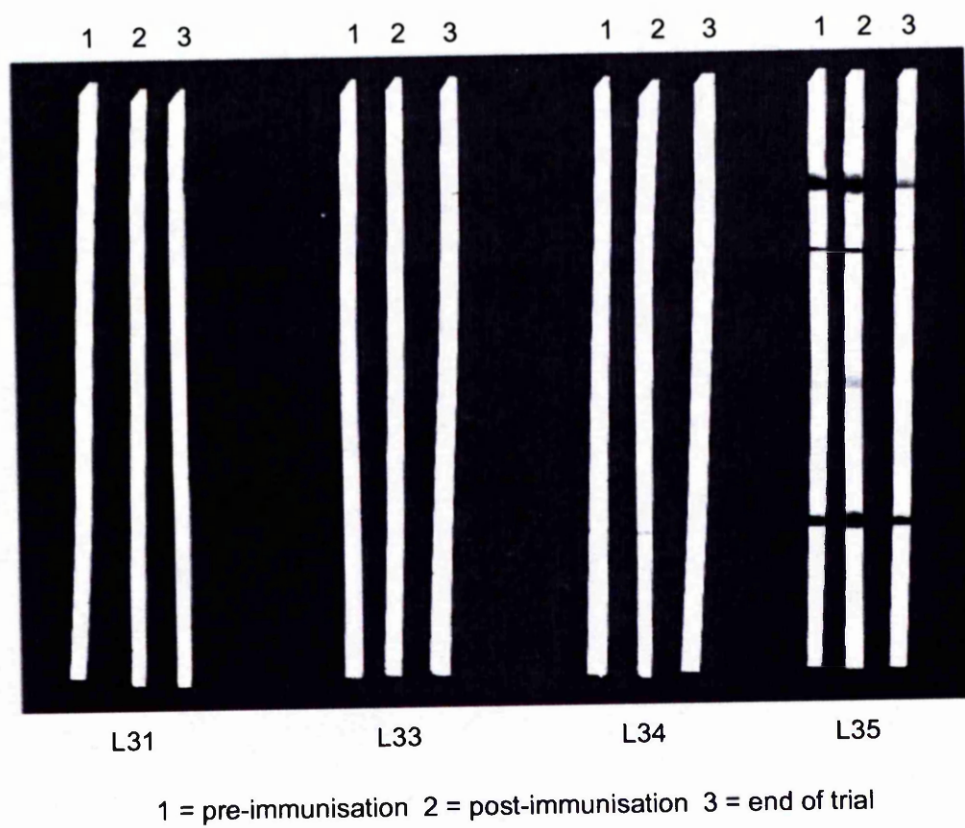
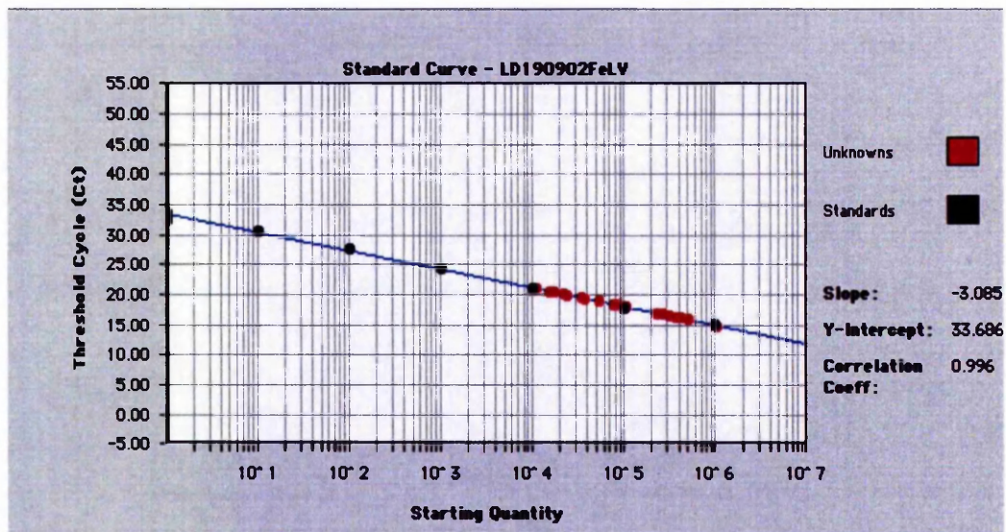


Figure 6-5 Quantitative real-time PCR Standard curves

FeLV proviral copy number assay



rDNA copy number assay

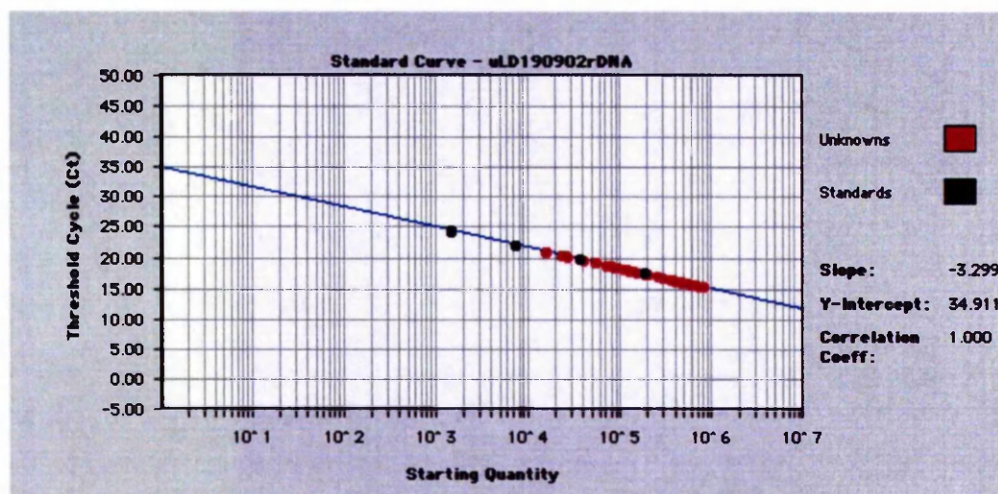


Figure 6-6 Log₁₀ of mean FeLV proviral copies per cell during trial

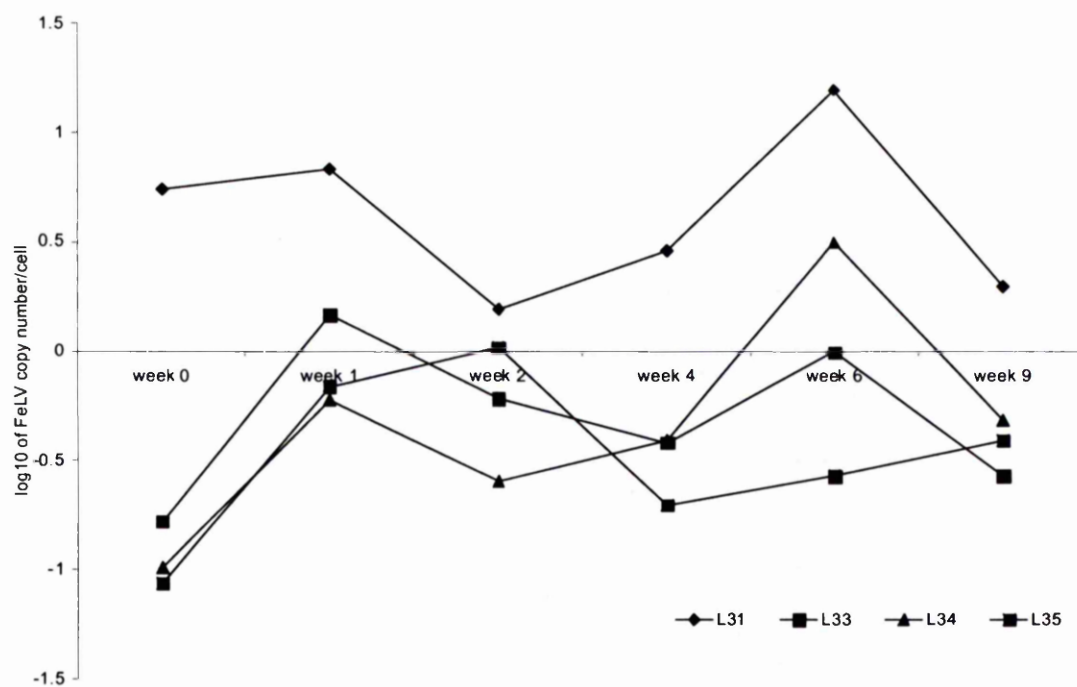


Figure 6-7 ELISA S/P ratios for animals during immunotherapy trial

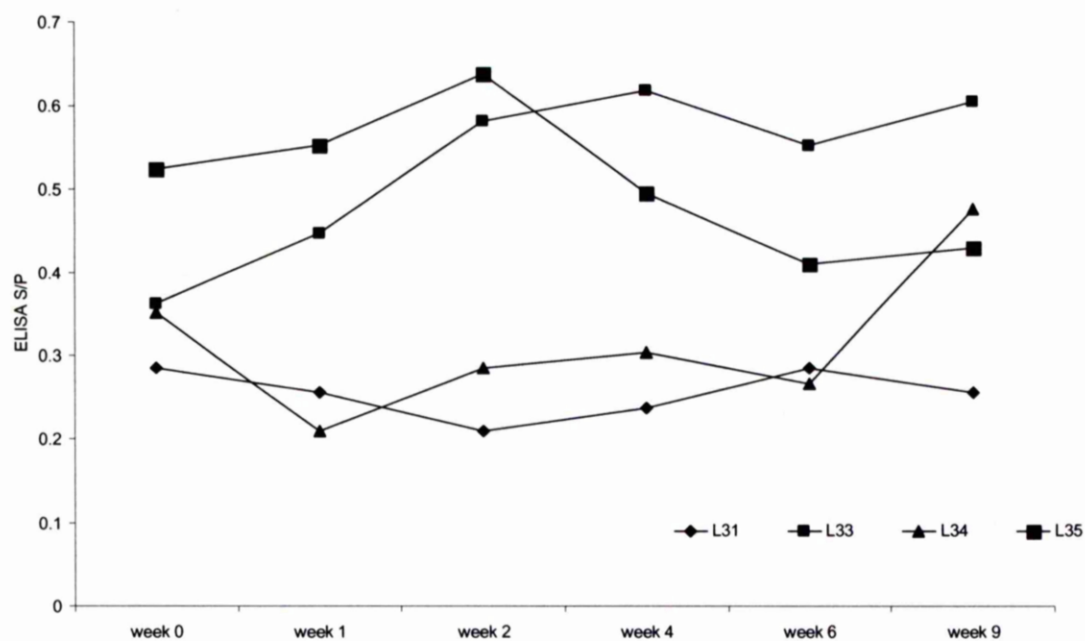
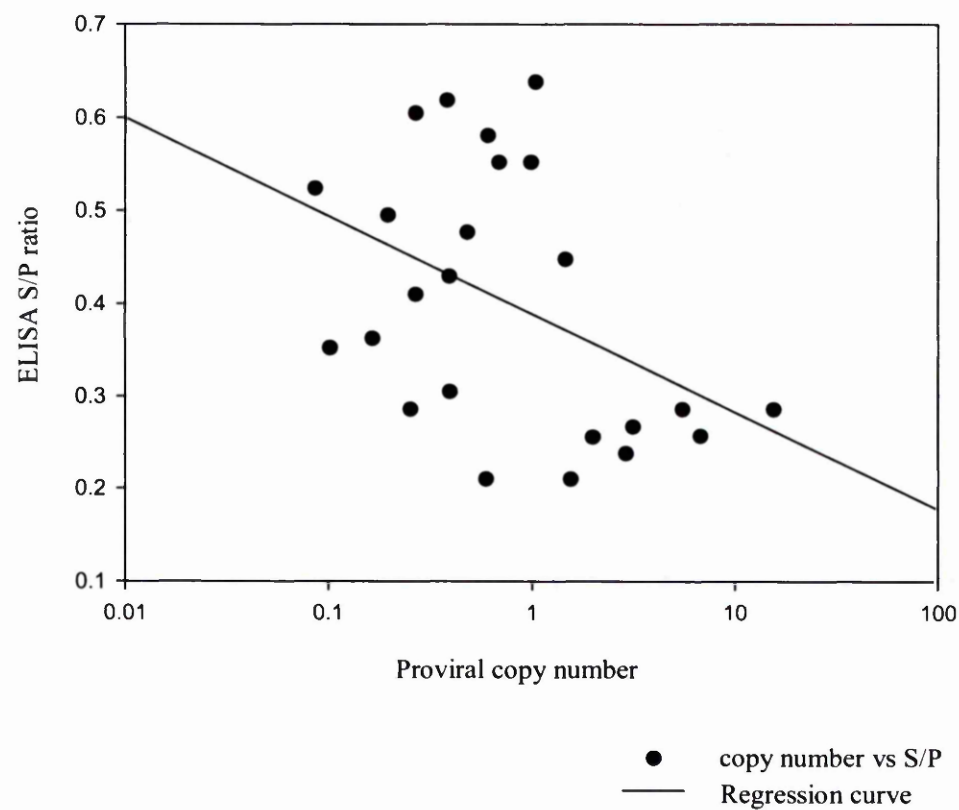


Figure 6-8 Correlation curve of ELISA S/P ratio and proviral copy number values



6.4 DISCUSSION

6.4.1 FeLV DNA vaccine and ILRAP-IL-18 as an immunotherapeutic agent

The combination of parameters measured during this study suggested that the use of an FeLV DNA vaccine with ILRAP-IL-18 was an ineffective immunotherapeutic agent for persistently viraemic animals. This vaccine was, however, extremely effective in providing protection of FeLV naïve cats against oronasal viral challenge, as shown in Chapter 5. In an attempt to explain this, a comparison must be made between the clinical and immune status of animals entering the vaccination trial and those used for immunotherapy.

Animals used for the vaccination trial were specific pathogen free cats which were exposed to a challenge of 4×10^6 f.f.u. in 4 equal doses over alternate days. As this challenge was applied oronasally, it is likely that only a proportion of the virus travelled across the mucous membranes and was exposed to the immune system of vaccinated cats. Animals used in the immunotherapy experiment however had high FeLV viral loads disseminated throughout the body produced by cells undergoing replication: FeLV can be isolated from many systems in viraemic cats including nasal passages, trachea, pharynx, intestines, bladder, pancreas, oesophagus and salivary glands [Rojko *et al.* 1979a]. In addition, persistently infected cats are highly contagious and have been shown to release virus in large quantities [Hardy *et al.* 1973a; Jarrett *et al.* 1973]. Therefore these viraemic animals would have been acting as a source of infection to each other and would have produced a cycle of continual natural challenge. It is likely then, that a disseminated, constantly cycling infection with high viral load, would pose a greater challenge to the DNA vaccine than a series of low dose, isolated viral exposures administered in the vaccination trial.

Another important difference between these experiments is the timing at which immunisation took place. In the vaccine trial, animals were challenged three weeks after the last vaccination. This allowed time for priming of the immune system with FeLV-specific antigen prior to viral exposure. The immune system in animals used in

the immunotherapy study had already been exposed to viral challenge in the vaccination trial and had failed to eliminate the virus. Therefore as an immunotherapeutic agent, the DNA vaccine will have been confronted by an established infection.

It has been shown that immune protection using this DNA vaccine is associated with the development of CTLs but not VNAb [Flynn *et al.* 2000a; Hanlon *et al.* 2001]. It follows therefore, that the vaccine may be effective against initial infection where CTLs are able to mount a response against a small number of FeLV infected cells. In persistently infected animals however, a much larger number of FeLV infected cells are present, both in the blood and in a variety of other systems [Rojko *et al.* 1979a]. Also, a large amount of free virus is released, requiring the development of VNAb [Russell and Jarrett, 1978a]. This DNA vaccine is therefore less likely to be effective in persistently infected cats.

Persistent infection with FeLV has been shown to induce dysfunction of the immune system. Studies have shown that kittens infected neonatally with FeLV, displayed atrophy of the thymus and depletion of other lymphoid organs [Anderson *et al.* 1971]. Kittens infected with FeLV at birth were shown to have depression of cell mediated immunity, measured by allograft retention times [Perryman *et al.* 1972]. In addition, Flynn *et al.* demonstrated a dramatically lower level of humoral and cell mediated immune mechanisms in cats which became persistently infected after FeLV challenge. This study found that FeLV-specific CTLs were detectable in the blood as early as 1 week post-challenge in FeLV recovered cats whereas CTL activity was undetectable in persistently infected cats until between 4 and 7 weeks after challenge. This CTL response then declined in viraemic cats but persisted until recovery in immune individuals [Flynn *et al.* 2002]. Animals infected with FeLV were also found to have defects in T and B cells. T cell defects were demonstrated by a decline in mitogen-induced blast transformation beginning at 5 weeks post-challenge until death at 24 weeks [Cockerell *et al.* 1976]. It is therefore probable that the immune system of the viraemic animals undergoing immunotherapy were compromised compared to those used in the vaccination trial despite the vaccinates having an immature, less developed immune system. Therefore, plasmid-encoded antigen may have been

presented successfully to the immune system, but immunodeficiency may have inhibited any detectable change in viral activity.

6.4.2 Parameters used to assess viral activity

It is important in the development of antiviral therapy that the viral status of the host is measured to allow an assessment of drug efficacy. However the level of viral infection comprises a combination of several dynamic processes, all of which should be taken into account when monitoring viral status. In the case of retroviruses, replication takes place once proviral DNA is integrated into the DNA of the host. This is then transcribed to produce mRNA in the host cell, which is processed to allow the generation of new virus and release from the cell [Fields and Knipe, 1990]. As described in 6.1.2, there are several ways in which this cycle can be quantified. Firstly, the proviral copy number per cell can be estimated [Flynn *et al.* 2002]. In addition, the level of RNA transcription has been used as a measurement of viral activity [Bagnarelli *et al.* 1994]. Finally, a common method used to assess retroviral load is the quantification of viral RNA in the plasma [Spector *et al.* 1998; Goto *et al.* 2002]. In general, these parameters have been found to correlate with the clinical status. For example, persistently infected cats were shown to have higher proviral copy numbers per PBMC than FeLV-recovered animals [Flynn *et al.* 2002]. However some studies have demonstrated differences between the plasma retroviral load and the level of viral RNA in virus-expressing cells [Hockett *et al.* 1999].

An assessment of FeLV plasma load may be a more accurate method of analysing the effect of immunotherapy in persistently infected cats. A decrease in proviral copy number per cell reflects the death of infected cells and replacement by healthy cells. This may be brought about by apoptosis from a cell mediated immune response, or cells reaching the end of their life span, which is dependent on the cell half life. This can be highlighted by data produced for HIV-1 where one study showed that the half-life of cell-free virus in plasma was approximately 6-fold less than the half life of productively infected cells [Perelson *et al.* 1996]. Therefore plasma viral load will decrease more rapidly in response to a change in viral activity than the level of viral infection of cells. However, as samples were only taken at weekly intervals, it is

unlikely that the assessment of viral plasma levels would have made any significant difference to the data collected. The fact that plasma was collected as well as buffy coat cells at these times, would allow assessment of viral RNA load at a later date to compare these parameters.

The levels of mean FeLV proviral copy numbers per cell varied between 15.537 and 0.058. With the exception of animal L31, the proviral loads were extremely low and similar levels of provirus were demonstrated in a previous study [Flynn *et al.* 2002]. This could represent an overall low level of integration in a high proportion of cells, or a high integration rate in a small proportion of cells. To investigate the frequency of proviral integration further, a study into the proportion of cells containing provirus could be performed.

6.5 Conclusion

This study was a preliminary experiment to assess the value of immunotherapy of persistently infected cats with a vaccine previously found to be efficacious in the prophylaxis of FeLV infection. A protocol using three weekly immunisations of DNA vaccine with ILRAP-IL-18 was found to have no effect on the parameters used to assess FeLV viral activity. However, in order to fully establish whether the pursuit of immunotherapy in this system is worthwhile, a fully controlled experiment using larger group sizes should be carried out using a reliable assay to quantify the level of FeLV in the plasma.

CHAPTER 7: GENERAL DISCUSSION

7 GENERAL DISCUSSION

7.1.1 Introduction

The aim of this project was to investigate further the role of the feline cytokines IL-12 and IL-18 as adjuvants to a DNA vaccine for FeLV. The previous study performed by Hanlon *et al.* identified a successful vaccine combination which provided complete protection against viraemia and a significant degree of protection from latent proviral infection. This combination comprised FeLV DNA vaccine in conjunction with feline IL-12 and PsecI signal feline IL-18 [Hanlon *et al.* 2001].

The work described here has involved the refinement of these constructs, potentially overcoming problems faced by both cytokines in their molecular structure. The development of feline specific polyclonal antibodies allowed conclusive evidence of *in vitro* protein expression of each cytokine. In addition, the development of bioassays for both IL-12 and IL-18 permitted the *in vitro* activity of these molecules to be established prior to their use in an *in vivo* situation. These cytokines were then used in an experimental study to investigate the relative roles of each cytokine in providing an adjuvant effect to a DNA vaccine described previously [Hanlon *et al.* 2001]. The potential immunotherapeutic use of the successful vaccine combination was subsequently assessed using persistently viraemic animals.

7.1.2 Interleukin-12

In recent years, the cloning of immune stimulating molecules such as IL-12 has facilitated a greater understanding of their function in the immune pathway and as a result their potential use in certain systems has been realised. As well as stimulating the proliferation and cytotoxicity of T and NK cells [Kobayashi *et al.* 1989; Wolf *et al.* 1991; Chehimi *et al.* 1993], the most vital biological function of IL-12 lies in its ability to stimulate the production of IFN γ from T cells [Marshall *et al.* 1995]. IFN γ belongs to the interferon family, a group of molecules related by their ability to protect cells from viral infection [Farrar and Schreiber, 1993]. IFN γ induces the production of certain molecules which inhibit the lifecycle of viral pathogens. IL-12 is

therefore able to induce a cascade effect which not only serves to enhance the cell-mediated branch of the immune system, but also to inhibit the replication of the pathogens themselves.

The primary objective in the development of a vector expressing IL-12 was to produce a plasmid capable of producing the bioactive heterodimer on transcription and translation. As described in 3.1.8, various constructs have been described including the use of separate plasmids encoding p35 and p40 [Hanlon *et al.* 2001], a single vector under the same or separate promoters [Tsuji *et al.* 1997; Yamakami *et al.* 2001], or the use of an IRES IL-12 construct [Zitvogel *et al.* 1994]. However, none of these vectors are guaranteed to prevent the overproduction of p40, which in homodimer formation is capable of inhibiting the activity of the heterodimer [Gillesen *et al.* 1995; Ling *et al.* 1995]. Another construct which ensures equimolar subunit products has been cloned in the pig. Here the subunits are linked by cDNA encoding the FMDV self-cleaving peptide, 2A. This sequence allowed cleavage of the polypeptide into separate subunits on translation and baculovirus vectors expressing this construct were found to produce bioactive IL-12 [Kokuho *et al.* 1999]. The presence of free p40 subunit however still allows the possibility of homodimer formation and antagonism of the bioactive molecule. The design of flexi-IL-12 ensures equimolar production of each subunit with the prevention of free p40 and has been proven to be active *in vitro* and *in vivo* in several species [Foss *et al.* 1999; Lode *et al.* 1999; McMonagle *et al.* 2001]. In addition, this fusion protein reduces the size of the construct required to deliver IL-12. This may be particularly advantageous in situations where the size of vector is crucial, such as packaging into vaccine vectors. To the author's knowledge, this is the first time that feline flexi-IL-12 has been described and this construct has the potential to be of value in the treatment and prevention of feline disease.

Further to the cloning of cytokine constructs, *in vitro* bioactivity data is crucial. This is particularly relevant where commercial therapy is viable, where batch testing of bioactivity would be required. The development of a simple, robust and reliable bioassay for IL-12 allows confirmation of activity and is useful to compare activity of new constructs in the field. In this work, an assay previously used for detection of equine IL-12 [McMonagle *et al.* 2001], was also found to be sensitive to feline

protein. This assay has proven to be relatively simple and reliable, relying on the dose dependent production of IFN γ from equine lymphocyte populations from lymph nodes collected at post mortem. A disadvantage of this system is that lymphocytes collected from different individuals showed differential sensitivity to IL-12, so standardisation of this assay was not possible.

IL-12 has been found to exert beneficial immune based effects in a variety of systems. This cytokine can increase immunity and survival rates of various pathogens such as *Toxoplasma gondii* [Khan *et al.* 1994], *Mycobacterium tuberculosis* [Cooper *et al.* 1995], *Leishmania major* [Heinzel *et al.* 1993] and MuLV [Gazzinelli *et al.* 1994]. In all these cases, it is the stimulation of Th1 responses and in some cases the inhibition of Th2 responses which elicit the protective effects from the pathogens concerned. In the case of FeLV, although virus neutralising antibodies tend to correspond with immunity [Hardy *et al.* 1976], it is clear that cell mediated immunity is also important. The presence of neutralising antibodies is not essential for viral protection [Jarrett 2001] and in the previous FeLV immunisation experiment, DNA vaccine immunisation did not produce neutralising antibodies but elicited protection from challenge. Additionally, protected animals showed increased levels of FeLV specific CTLs compared to control animals [Flynn *et al.* 2000a; Hanlon *et al.* 2001]. It could be hypothesised then, that delivery of IL-12 would be beneficial in mounting an immune response against FeLV due to its ability to stimulate cell mediated immunity. Data produced from this *in vivo* study however suggests that in this system, IL-12 does not enhance the level of protection elicited in cats against a DNA vaccine for FeLV. A similar result was also produced in the previous trial, where the adjuvancy of IL-12 was analysed using administration of separate p35 and p40 plasmids [Hanlon *et al.* 2001]. This is suggestive that the IL-12 fusion protein does not in this case offer any significant advantage over separate plasmids in its *in vivo* adjuvant effect. As suggested in 5.4.5, it is possible that the dose [Orange *et al.* 1994], timing and site of administration [Chen *et al.* 2001], may have affected the *in vivo* response to IL-12, parameters which have been shown to be important in other IL-12 studies.

7.1.3 Interleukin-18

IL-18 is another cytokine which has shown potential in the therapeutic manipulation of the immune response. Similar to IL-12 however, an IL-18 construct must bring about efficient bioactive protein production and successful secretion from the cell. As detailed in 4.1.3, pro-IL-18 construct produces the precursor protein which is inactive unless it is cleaved by natural caspase-1 and mature-IL-18 protein although bioactive, lacks the natural signal peptide for cell secretion. These problems were overcome using a synthetic signal peptide derived from the human signal sequence of the IL-1 β receptor antagonist protein (ILRAP) gene which allowed feline mature-IL-18 to be transferred across the cell membrane. This construct showed superior levels of *in vitro* expression and bioactivity when compared with the previously used feline PsecI-IL-18 construct [Hanlon *et al.* 2001]. Again, this data is of importance as IL-18 may be of commercial value in feline medicine in the future.

As progress is made in terms of investigation and clinical trials into IL-18, the specific quantification and activity of the molecule in blood and other tissues is a prerequisite to further development. Until recently, the dose dependent production of IFN γ from PBMCs was used to detect bioactive IL-18 [Ushio *et al.* 1996]. This system was not necessarily specific to IL-18 and was time consuming to perform. The KG-1 bioassay however [Konishi *et al.* 1997], proved to be simple and reliable, producing consistent results throughout this study. Its specificity was confirmed by the suppression of bioactivity using IL-18 neutralising antibody. This antibody did not produce complete suppression of IFN γ production from cells whereas the negative control transfection samples demonstrated background levels of bioactivity. This suggests that either the antibody level was not sufficiently high to bind all IL-18 molecules, or that other factors, induced by IL-18 were stimulating IFN γ production.

7.1.4 Vaccination trial

The use of IL-18 expression vectors *in vivo* have been found to be efficacious in a variety of systems including tumour therapy [Osaki *et al.* 1999], therapy against pathogens [Tanaka-Kataoka *et al.* 1998] and as adjuvants to DNA vaccination

[Hanlon *et al.* 2001]. Similarly this project has demonstrated that the feline synthetic signal IL-18 dramatically increases the level of immune protection to viral challenge compared to FeLV DNA vaccine alone. This experiment was designed to investigate the relative roles of IL-12 and IL-18 constructs as adjuvants to a DNA vaccine for FeLV used previously [Hanlon *et al.* 2001]. The protective effect of each interleukin alone and in combination was analysed by its ability to protect animals from FeLV viraemia and latency. The DNA vaccine with ILRAP-IL-18 as an adjuvant stimulated complete protection from viraemia where 4 of 6 control animals became persistently viraemic. Latency data demonstrated that only 1 of 6 cats were latently infected in the vaccine and ILRAP-IL-18 group, whereas all animals showed bone marrow infection in the control group.

The gravity of this result can only be fully appreciated when comparisons are made with FeLV vaccines currently commercially available. Previous FeLV vaccine data shows that very few studies have shown complete protection of animals against viraemia. Lafrado [1994] demonstrated complete protection in cats vaccinated with Leucocell 2, but only 19% of control animals were viraemic, suggesting that the viral challenge used was not effective. An experiment elicited complete protection from viraemia using Fel-O-Vax [Legendre *et al.* 1991]. However at euthanasia 42% animals were latently infected with provirus. Sebring *et al.* [1991] also demonstrated complete protection using Fel-O-Vax, but unfortunately latent infection was not analysed in this study. The data produced from this study appears to be superior to all FeLV vaccine studies published to date.

The DNA vaccine combined with ILRAP-IL-18 and flexi-IL-12 also elicited complete protection from FeLV, but a higher proportion of these animals were latently infected with provirus (2 of 6 cats). A similar result was produced in the previous DNA vaccine trial using IL-12 delivered in separate plasmids and PsecI-IL-18, with complete protection from viraemia with one latently infected animal [Hanlon *et al.* 2001]. Although this group displayed inferior protection in comparison to ILRAP-IL-18 adjuvant alone, the result still compares favourably with studies of the vaccines commercially available.

In conclusion, this study shows that the use of the feline ILRAP-IL-18 in combination with a FeLV DNA vaccine demonstrated potential as an effective vaccine against viraemia and latent infection, comparing favourably with the existing commercial vaccines currently available. This data confirms the result produced by Hanlon *et al.*[2001], where complete protection against viraemia and some protection against latent infection were shown by the combination of DNA vaccine, IL-12 and IL-18. The development of improved constructs flexi-IL-12 and ILRAP-IL-18 did not improve the adjuvant effect of the previously used cytokine constructs. However this experiment identifies the IL-18 component as the important vaccine adjuvant, which alone can induce significant protection against challenge. Furthermore, this vaccine combination was shown to be efficacious against oronasal challenge with FeLV, a more natural form of challenge than the intraperitoneal route used previously [Hanlon *et al.* 2001].

7.1.5 Immunotherapy trial

The FeLV vaccine with ILRAP-IL-18 was then used in an immunotherapy study, where four persistently infected cats were inoculated three times at weekly intervals. This was to establish if immunisation could influence the viral load in tissues of animals with established FeLV infection. The data produced suggested that immunotherapy with this vaccine combination had no beneficial effect on the viral status of persistently infected cats. Animals remained viraemic throughout the study and the level of FeLV proviral DNA levels in buffy coat cell samples after immunisation were similar to levels prior to inoculation.

7.1.6 Future work

This project has produced *in vitro* evidence which shows that both bioactive flexi-IL-12 and ILRAP-IL-18 are expressed at high levels within cells. These constructs were used as cytokine adjuvants to an FeLV DNA vaccine and were found to be highly efficacious *in vivo*. The combination of DNA vaccine and ILRAP-IL-18 was particularly effective and compared extremely favourably to commercial vaccines currently available. A major constraint to this *in vivo* experiment however was the

number of animals used in each vaccination group, which limited statistical analysis. Therefore in order to examine the possibility of commercialisation of this vaccine, a trial comprising larger vaccine groups should be performed. It would also be beneficial include the addition of persistently infected animals from viral challenge onwards to reproduce natural FeLV infection as fully as possible. Of particular interest in this study would be the inclusion of some of the most efficacious commercial vaccines which would allow a fair comparison of each vaccine under the same conditions. Future work into feline flexi-IL-12 would require investigation *in vivo* into the dose, timing and site of administration of the IL-12 construct in comparison to the DNA vaccine.

In addition, the beneficial effects which the feline ILRAP-IL-18 construct has displayed in this experiment holds potential in other areas of feline medicine such as tumour therapy and immunotherapy against other pathogens.

This work combined with the previous FeLV DNA vaccination study [Hanlon *et al.* 2001], shows great potential for the generation of a new vaccine for FeLV. Factors which must be considered for commercialisation include the cost of production and storage conditions. One of the main advantages of DNA vaccines is their cheap, simple and efficient production. Furthermore, this type of vaccine is heat stable and does not require the cold chain which is necessary for other more traditional vaccines. However, there are some potential problems which would have to be overcome in order to make commercialisation a viable option. The means of delivery of DNA such as multiple administration and the requirement of naked skin in the case of gene gun administration, poses a challenge in DNA vaccination of domestic animals. In addition, the variability in the individual response and the issue of safety regarding DNA vaccination also needs to be addressed before DNA vaccines can become commercially available. However, if future studies on this vaccine were to confirm the results produced in this work, then there would be a strong case for commercialisation of this vaccine in the future.

GENERAL ABBREVIATIONS

GENERAL ABBREVIATIONS

$^{\circ}\text{C}$	degrees Celsius
μl	microlitres
μM	micromolar
2-ME	β -mercaptoethanol
A	adenine or adenosine
aa	amino acid
Ab	antibody
AcPL	accessory protein-like protein
AEBSF	4-(-aminoethyl)benzenesulphonyl fluoride
Ag	antigen
AIDS	acquired immunodeficiency syndrome
AML	acute myeloid leukaemia
AP	alkaline phosphatase
APC	antigen presenting cell
APS	ammonium persulphate
ATP	adenosine triphosphate
BCG	<i>Mycobacterium bovis</i> bacillus Calmette-Guerin
Bas	basophil
BGH	bovine growth hormone
BM	bone marrow
B Neut	band neutrophil
bp	base pair
BSA	bovine serum albumin
C	cytosine or cytidine
CB	caspase buffer
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
CO_2	carbon dioxide
CSF	colony stimulating factor
CTL	cytotoxic T lymphocyte
Da	dalton

DC	dendritic cells
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
DTH	delayed-type hypersensitivity
EAE	experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
enFeLV	endogenous FeLV-related sequences
Eos	eosinophil
EU	endotoxin units
FBS/FCS	foetal bovine serum/foetal calf serum
FeLV	feline leukaemia virus
FeSV	feline sarcoma virus
f.f.u.	focus forming units
FIV	feline immunodeficiency virus
FMDV	foot-and-mouth disease virus
FOCMA	feline oncornavirus-associated cell membrane antigen
g	grams
G	guanine or guanosine
GCG	Genetics Computer Group
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HA	haemagglutinin
Hb	haemoglobin
HCT	haematocrit
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N</i> '-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
HTLV	human T-cell leukaemia virus

ICE	interleukin-1 β converting enzyme
ICSBP	interferon consensus sequence binding protein
ID	intra dermal
IFN α	interferon alpha
IFN γ	interferon gamma
Ig	immunoglobulin
IGIF	interferon gamma inducing factor
IL-12	interleukin 12
IL-18	interleukin 18
IL-1Rrp	IL-1 receptor related protein
IL-12R	IL-12 receptor
IL-18BP	IL-18 binding protein
ILRAP	IL-1 β receptor antagonist protein
IM	intramuscular
IP	intraperitoneal
IPTG	isopropyl β -D-1-thiogalactopyranoside
IRAK	IL-1 receptor-activating kinase
IRES	internal ribosome entry site
ISCOM	immunostimulating complex
IU	international unit
IV	intravenous
JAK	Janus-family kinase
JEV	Japanese encephalitis virus
JNK	c-Jun NH ₂ -terminal kinase
kb	kilobase
kDa	kilodalton
L	litre
LAL	Limulus amoebocyte lysate
LB	Luria Bertani medium
LPS	lipopolysaccharide
LTR	long terminal repeat
Lymph	lymphocyte
M	molar

MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
ME	mercaptoethanol
mg	milligram
MHC	major histocompatibility complex
MIDGE	minimalistic immunogenic defined gene expression vector
mM	millimolar
Mono	monocyte
mRNA	messenger ribonucleic acid
Neut	neutrophil
NF- κ B	nuclear factor - κ B
NK	natural killer cell
NP	nucleoprotein
NTP	nucleoside triphosphate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF	preventable fraction
PHA	phytohaemagglutinin
PV	persistent viraemia
r	recombinant
RBC	red blood cell
rDNA	ribosomal DNA
RIM	rapid immunomigration
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RSV	respiratory syncytial virus, Rous sarcoma virus
RT	reverse transcriptase

RT-PCR	reverse transcriptase polymerase chain reaction
SC	subcutaneous
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
S/P	sample to positive
SPF	specific pathogen free
STAT	signal transducers and activators of transcription
SV40	Simian virus 40
T	thymine or thymidine
TBE	tris/borate/EDTA
TBS	tris buffered saline
TBS-T	tris buffered saline-tween solution
TCR	T cell receptor
TEMED	N'-tetramethyl-ethylenediamine
Th1	T helper cell type 1
Th2	T helper cell type 2
TK	thymidine kinase
TLR	toll-like receptor
TMB	3,3', 5,5'-tetramethyl-benzene
TNF α	tumour necrosis factor-alpha
TRAF6	tumour necrosis factor receptor associated kinase
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris hydroxychloride
UV	ultraviolet
VI	virus isolation
VNAb	virus neutralising antibody
NNAb	non-neutralising antibody

WBC	white blood cell
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

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APPENDIX

APPENDIX: Haematology data

Abbreviations used in tables:

ABBREVIATION	FULL NAME	NORMAL VALUE
RBC	Total red blood cell count x 10 ¹² /L	5.0-10.0 x 10 ¹² /L
Hb	Haemoglobin, g/L	10.0-15.0g/L
HCT	Haematocrit, or packed cell volume (PCV)	30-45ml/L
MCV	Mean red cell volume	39.0-55.0fl
MCH	Mean red cell haemoglobin	12.5-17.5pg
MCHC	Mean red cell haemoglobin concentration	30.0-36.0g/dl
WBC	Total white blood cell count x 10 ⁹ /L	5.5-15.5 x 10 ⁹ /L
B NEUT	Immature (band) neutrophil count x 10 ⁹ /L	rare
NEUT	Neutrophil count x 10 ⁹ /L	2.5-12.5 x 10 ⁹ /L
LYMPH	Lymphocyte count x 10 ⁹ /L	1.5-7.0 x 10 ⁹ /L
MONO	Monocyte count x 10 ⁹ /L	0.00-0.85 x 10 ⁹ /L
EOS	Eosinophil count x 10 ⁹ /L	0.0-1.5 x 10 ⁹ /L
BAS	Basophil count x 10 ⁹ /L	rare

Pre-trial Haematology results

CAT	DATE	RBC	Hb	HCT	MCV	MCH	MCHC	WBC	B Neut	Neut	Lymph	Mono	Eos	Bas
L1	7/1/02	6.18	9.3	24.6	39.9	15.0	37.7	12.4	0.1	8.308	3.348	0.496	0	0.124
L2	7/1/02	6.18	9.8	25.5	41.2	15.9	38.5	15.9	0	9.699	4.77	0.318	1.113	0
L3	7/1/02	7.39	10.1	26.7	36.2	13.7	37.9	12.8	0	7.808	4.608	0.256	0.128	0
L4	7/1/02	6.69	10.6	27.8	41.5	15.8	38.1	8.54	0	5.466	2.135	0.342	0.598	0
L5	7/1/02	6.44	9.8	25.9	40.2	15.2	37.8	14.6	0	8.322	5.256	0	1.022	0
L6	7/1/02	6.16	9.94	26.3	42.7	16.2	37.8	13.7	0	9.727	2.603	0.411	0.822	0.137
L8	7/1/02	7.54	12.1	31.3	41.5	16.0	38.6	13.8	0	8.97	4.14	0.414	0.276	0
L9	7/1/02	7.79	10.5	27.6	35.4	13.5	38.1	14.7	0	10.143	2.793	0.882	0.882	0
L10	7/1/02	6.2	9.24	24.4	39.3	14.9	37.9	14.3	0	9.438	2.717	1.001	1.144	0
L11	7/1/02	6.35	10.1	26.3	41.4	15.9	38.3	14.0	0	9.1	3.78	0.14	0.98	0
L12	7/1/02	7.02	9.7	26.0	37.0	13.8	37.4	18.9	0	13.041	5.481	0.189	0	0.189
L13	8/1/02	6.93	9.49	25.9	37.4	13.7	36.7	10.5	0	5.355	4.41	0.21	0.525	0
L14	8/1/02	7.48	10.7	28.8	38.5	14.4	37.3	9.48	0	5.404	2.086	0.19	1.801	0
L15	8/1/02	6.51	9.88	26.1	40.1	15.2	37.8	7.47	0	3.959	3.137	0.075	0.299	0
L16	8/1/02	6.41	10.0	26.3	41.0	14.7	38.2	12.8	0	7.68	3.456	0.384	1.152	0.128
L17	8/1/02	6.75	10.4	26.2	38.8	15.4	39.7	8.91	0	4.455	3.831	0.267	0.356	0
L18	8/1/02	6.44	10.0	26.0	40.3	15.6	38.6	13.7	0	9.179	3.014	0.411	0.959	0.137
L19	8/1/02	6.64	9.94	26.0	39.1	15.0	38.3	28.5	0	23.085	3.705	1.425	0.285	0
L20	8/1/02	6.61	10.2	26.8	40.5	15.4	38.1	22.7	0	15.436	6.129	0.454	0.681	0
L21	8/1/02	6.29	9.74	25.8	41.0	15.5	37.8	6.84	0	2.804	3.215	0.205	0.616	0
L22	8/1/02	5.75	9.63	25.7	44.8	16.8	37.4	12.9	0	7.998	3.483	0.129	1.29	0
L23	8/1/02	7.07	9.37	24.9	35.2	13.2	37.7	14.4	0	9.072	3.744	0.432	1.152	0
L24	8/1/02	6.5	9.66	26.1	40.1	14.9	37.0	19.2	0	14.4	2.88	0.96	0.576	0.384
L25	9/1/02	7.02	9.42	25.1	35.8	13.4	37.5	6.98	0	4.258	1.605	0.209	0.838	0.07
L26	9/1/02	7.83	9.92	26.8	34.3	12.7	37.0	13.6	0	7.888	3.672	0	1.904	0.136
L27	9/1/02	5.34	8.93	23.5	44.0	16.7	38.0	23.3	0	17.242	3.262	1.165	1.165	0.233
L28	9/1/02	6.54	10.1	26.8	41.0	15.5	37.8	19.9	0	13.352	5.373	0.199	0.597	0.199
L29	9/1/02	6.32	9.58	24.8	39.3	15.2	38.5	13.7	0	7.535	4.658	0.685	0.822	0
L30	9/1/02	6.62	9.54	25.6	38.7	14.4	37.2	11.0	0	8.25	1.65	0.33	0.77	0
L31	9/1/02	7.08	11.2	29.4	41.5	15.9	38.2	19.9	0	13.333	4.975	0.597	0.796	0.199
L32	9/1/02	6.90	10.3	26.5	38.4	14.9	38.7	14.6	0	8.468	5.402	0	0.584	0.146
L33	9/1/02	6.04	9.69	25.0	41.4	16.0	38.8	13.8	0	7.176	5.382	0.276	0.828	0.138
L34	9/1/02	7.22	10.1	27.1	37.5	14.1	37.5	13.9	0	6.95	5.143	0.556	1.112	0.139
L35	9/1/02	5.86	9.45	23.5	40.1	16.1	40.2	10.3	0	6.18	2.781	0.515	0.824	0
L36	9/1/02	7.39	11.1	28.4	38.5	15.0	39.0	11.0	0	0.11	6.82	3.19	0.33	0.55

Haematology results on day of challenge

CAT	DATE	RBC	Hb	HCT	MCV	MCH	MCHC	WBC	B Neut	Neut	Lymph	Mono	Eos	Bas
L1	25/2/02	9.01	12.4	32.7	36.3	13.8	38.1	9.64	0	4.916	4.627	0	0.096	0
L2	25/2/02	7.15	11.5	29.2	40.8	16.1	39.5	14.2	0	7.81	3.834	0.568	1.846	0.142
L3	25/2/02	7.97	11.3	28.7	36.0	14.1	39.2	11.0	0	4.51	6.38	0	0.11	0
L4	25/2/02	6.81	11.1	28.9	42.4	16.3	38.4	7.02	0	3.721	2.948	0.07	0.281	0
L5	25/2/02	6.67	10.4	25.9	38.8	15.6	40.1	13.0	0	4.68	7.28	0	0.91	0.13
L6	25/2/02	7.30	12.3	31.1	42.6	16.9	39.7	11.9	0	6.902	3.808	0.357	0.833	0
L8	25/2/02	8.14	13.1	33.9	41.6	16.0	38.6	8.55	0	4.788	3.164	0.257	0.257	0.086
L9	25/2/02	7.79	11.0	27.3	35.0	14.2	40.5	11.6	0	6.264	3.944	0.58	0.812	0
L10	25/2/02	7.71	11.1	28.2	39.4	15.5	39.2	6.80	0	3.4	2.992	0	0.408	0
L11	25/2/02	7.94	11.2	29.2	36.8	14.1	38.3	7.35	0	7.579	6.006	0.429	0.286	0
L12	25/2/02	7.16	11.2	28.8	40.2	15.6	38.9	14.3	0	3.675	3.528	0.147	0	0
L13	26/2/02	8.18	11.0	29.8	36.4	13.5	37.0	7.53	0	4.141	2.937	0.151	0.226	0.075
L14	26/2/02	7.75	11.2	28.9	37.3	14.5	38.9	11.7	0	5.499	4.797	0.117	1.287	0
L15	26/2/02	6.91	10.4	27.4	39.6	15.0	37.9	10.3	0	4.944	4.841	0.206	0.309	0
L16	26/2/02	7.05	11.2	29.7	42.1	15.8	37.6	8.89	0	4.178	3.556	0.089	1.067	0
L17	26/2/02	7.66	11.2	30.0	39.1	14.6	37.3	12.0	0	6.36	5.28	0	0.36	0
L18	26/2/02	7.22	11.1	29.6	41.0	15.4	37.5	12.9	0	8.514	3.354	0.387	0.516	0.129
L19	26/2/02	7.56	11.3	30.0	39.7	15.0	37.6	14.1	0	7.191	6.345	0.282	0.282	0
L20	26/2/02	7.68	12.1	32.2	41.9	15.7	37.5	16.9	0	11.999	4.056	0.338	0.169	0.338
L21	26/2/02	7.22	10.7	28.6	39.6	14.8	37.5	13.3	0	6.783	5.719	0.266	0.532	0
L22	26/2/02	6.34	11.1	28.2	44.5	17.5	39.3	18.8	0	13.16	3.76	0.376	1.128	0.376
L23	26/2/02	7.78	11.2	28.8	37.0	14.4	38.9	18.8	0	12.22	3.572	0.188	2.632	0.188
L24	26/2/02	8.00	12.3	32.2	40.3	15.4	38.1	25.0	0	16	6	1.5	1.25	0.25
L25	27/2/02	8.04	10.9	29.9	37.2	13.6	36.4	11.6	0	6.496	4.176	0.232	0.696	0
L26	27/2/02	8.65	10.7	29.8	34.4	12.3	35.8	11.3	0	6.893	4.181	0	0.226	0
L27	27/2/02	6.84	10.9	30.1	44.0	16.0	36.3	19.7	0	13.79	4.728	0.197	0.788	0.197
L28	27/2/02	6.70	10.6	28.1	41.9	15.9	37.9	15.9	0	11.13	3.657	0	0.954	0.159
L29	27/2/02	7.46	11.1	29.4	39.4	14.8	37.7	11.7	0	6.201	4.563	0	0.936	0
L30	27/2/02	8.43	11.9	33.2	39.4	14.1	35.7	8.32	0	5.907	2.08	0.083	0.25	0
L31	27/2/02	7.97	12.6	33.3	41.8	15.8	37.8	16.1	0	8.694	6.762	0.322	0.322	0
L32	27/2/02	7.13	10.4	27.4	38.3	14.6	38.0	16.4	0	7.216	8.364	0.328	0.492	0
L33	27/2/02	6.38	10.3	26.5	41.5	16.1	38.7	14.6	0	7.008	6.132	0.438	1.022	0
L34	27/2/02	8.66	12.2	31.9	36.8	14.0	38.1	9.37	0	4.029	4.591	0.094	0.562	0.094
L35	27/2/02	7.19	10.9	28.7	40.0	15.2	38.0	21.8	0	14.17	5.45	0.436	1.744	0
L36	27/2/02	7.72	11.2	29.9	38.7	14.5	37.4	17.2	0	11.352	4.988	0.172	0.516	0.172

Haematology results at 6 weeks post-challenge

CAT	DATE	RBC	Hb	HCT	MCV	MCH	MCHC	WBC	B Neut	Neut	Lymph	Mono	Eos	Bas
L1	15/4/02	7.78	11.2	28.5	36.6	14.4	39.4	12.6	0	8.316	3.654	0.504	0	0.126
L2	15/4/02	7.10	11.3	30.1	42.4	15.9	37.4	10.4	0	5.616	3.12	0.832	0.832	0
L3	15/4/02	7.96	11.3	30.2	37.9	14.2	37.3	11.8	0	5.546	5.31	0.472	0.354	0.118
L4	15/4/02	6.97	11.1	29.6	42.5	16.0	37.5	4.55	0	2.366	1.729	0.137	0.319	0
L5	15/4/02	6.83	10.4	27.8	40.7	15.2	37.4	15.8	0	7.268	7.9	0.316	0.316	0
L6	15/4/02	8.17	13.5	36.1	44.2	16.6	37.5	9.54	0	5.629	3.244	0.191	0.382	0.095
L8	15/4/02	8.10	13.6	35.2	43.4	16.7	38.6	5.04	0	0.655	4.284	0.101	0	0
L9	15/4/02	8.13	11.5	30.0	37.0	14.2	38.3	8.47	0	4.066	3.388	0.254	0.678	0.085
L10	15/4/02	7.37	11.4	30.2	40.9	15.5	37.8	5.68	0	2.897	2.442	0.284	0.057	0
L11	15/4/02	7.14	11.2	29.4	41.2	15.8	38.3	6.09	0	2.984	2.862	0.061	0.183	0
L12	15/4/02	8.05	11.2	29.5	36.6	13.9	37.8	13.5	0	6.345	7.02	0	0.135	0
L13	16/4/02	8.58	11.9	30.9	36.0	13.9	38.5	12.6	0	4.284	7.434	0.378	0.504	0
L14	16/4/02	7.44	11.1	29.5	39.6	15.0	37.8	6.36	0	3.371	2.671	0.064	0.254	0
L15	16/4/02	7.40	11.1	28.9	39.0	15.1	38.6	13.7	0	5.069	7.809	0.548	0.274	0
L16	16/4/02	8.06	13.0	33.6	41.7	16.1	38.7	13.8	0	6.624	5.658	0.552	0.828	0.138
L17	16/4/02	7.62	11.6	30.1	39.5	15.3	38.6	11.2	0	5.152	5.264	0.224	0.56	0
L18	16/4/02	8.15	12.7	32.6	40.0	15.6	38.9	11.4	0	5.358	5.472	0.114	0.456	0
L19	16/4/02	7.37	11.7	29.6	40.2	15.9	39.5	13.9	0	10.008	3.892	0	0	0
L20	16/4/02	7.58	12.5	31.7	41.8	16.5	39.5	16.0	0	10.24	4	0.8	0.96	0
L21	16/4/02	7.83	11.9	31.7	40.5	15.2	37.4	5.94	0	2.376	3.089	0.178	0.238	0
L22	16/4/02	6.03	10.7	27.3	45.3	17.8	39.3	4.37	0	2.229	1.486	0.044	0.612	0
L23	16/4/02	8.06	12.1	31.0	38.5	15.0	39.1	11.3	0	7.232	2.825	0.339	0.904	0
L24	16/4/02	7.79	12.6	33.3	42.8	16.2	37.7	5.84	0	2.57	2.57	0.409	0.292	0
L25	17/4/02	7.84	10.7	28.1	35.9	13.6	38.0	6.72	0	3.226	2.554	0	0.941	0
L26	17/4/02	8.32	10.3	28.0	33.6	12.4	36.9	12.5	0	5.875	5.875	0.5	0.75	0
L27	17/4/02	7.25	11.6	30.0	41.1	16.0	38.6	16.1	0	7.889	7.245	0.161	0.805	0
L28	17/4/02	6.91	11.2	28.8	41.7	16.3	38.9	10.8	0	5.508	4.644	0	0.432	0.216
L29	17/4/02	8.03	12.1	31.4	39.1	15.1	38.6	6.21	0	1.553	3.974	0.186	0.497	0
L30	17/4/02	8.45	12.3	32.7	38.7	14.6	37.7	10.2	0	6.732	2.652	0.102	0.714	0
L31	17/4/02	8.17	13.1	33.8	41.4	16.0	38.7	13.6	0	6.936	5.712	0.136	0.816	0
L32	17/4/02	7.69	11.2	28.8	37.4	14.6	39.0	13.7	0	6.576	6.85	0	0.274	0
L33	17/4/02	6.59	11.0	28.3	43.0	16.7	38.9	12.7	0	4.826	7.366	0.381	0.127	0
L34	17/4/02	9.32	13.1	33.4	35.9	14.0	39.1	10.2	0	6.018	3.06	0.306	0.816	0
L35	17/4/02	6.88	11.0	27.4	39.8	15.9	40.1	17.6	0	12.848	3.872	0.704	0.176	0
L36	17/4/02	8.28	12.4	32.2	38.9	15.0	38.6	8.85	0	5.045	3.275	0.088	0.443	0

Haematology results at 12 weeks post-challenge

CAT	DATE	RBC	Hb	HCT	MCV	MCH	MCHC	WBC	B Neut	Neut	Lymph	Mono	Eos	Bas
L1	27/5/02	8.81	13	33.5	38	14.7	38.7	12.2	0	5.612	6.344	0.122	0.122	0
L2	27/5/02	7.94	13.1	33.5	42.2	16.5	39.1	12.1	0	7.26	3.388	0.121	1.21	0.121
L3	27/5/02	9.05	13.4	33.7	37.2	14.8	39.9	10.7	0	3.852	6.206	0.107	0.428	0.107
L4	27/5/02	7.53	12.3	31.9	42.4	16.4	38.7	7.93	0	4.044	3.013	0.238	0.634	0
L5	27/5/02	7.62	12.1	30.7	40.3	15.9	39.4	14.4	0	5.328	8.352	0.288	0.432	0
L6	27/5/02	9.02	15.5	39.9	44.3	17.2	38.9	11.7	0	6.435	4.446	0	0.702	0.117
L8	27/5/02	7.5	13.1	33.1	44.1	17.5	39.7	12.4	0	4.464	6.944	0.372	0.62	0
L9	27/5/02	9.6	14	35.8	37.3	14.6	39.3	8.54	0	4.441	2.733	0.256	1.11	0
L10	27/5/02	8.28	13.5	35.2	42.5	16.4	38.5	9.11	0	3.371	4.919	0.364	0.364	0.091
L11	27/5/02	6.67	11.3	29.8	44.6	17.0	38.1	11.7	0	4.68	6.552	0.234	0.234	0
L12	27/5/02	8.89	12.7	33.1	37.3	14.3	38.4	8.57	0	4.114	4.028	0.343	0.086	0
L13	28/5/02	8.61	11.7	31.2	36.2	13.5	37.4	5.17	0.052	1.706	2.895	0.207	0.259	0.052
L14	28/5/02	7.67	11.3	31	40.4	14.8	36.6	10.1	0	4.848	4.141	0.101	1.01	0
L15	28/5/02	7.98	11.8	31.9	40	14.8	37	12.9	0	3.612	8.643	0.516	0.129	0
L16	28/5/02	7.96	12.7	34.3	43	15.9	37	12.4	0	6.448	4.588	0.372	0.992	0
L17	28/5/02	7.65	11.7	30.8	40.2	15.3	38.1	8.56	0	4.879	3.338	0.086	0.171	0.086
L18	28/5/02	8.39	13.1	34.4	40.9	15.6	38	8.97	0	4.395	3.947	0.09	0.538	0
L19	28/5/02	7.55	11.5	30.7	40.6	15.2	37.5	10.3	0	3.811	6.18	0.103	0.206	0
L20	28/5/02	8.18	13.2	34.2	41.8	16.1	38.4	12.4	0	7.44	4.34	0.248	0.372	0
L21	28/5/02	7.8	12.1	31.8	40.8	15.6	38.1	8.94	0	4.649	3.397	0.447	0.447	0
L22	28/5/02	7.16	12.9	35	48.9	18.1	36.9	8.13	0	2.52	4.878	0.244	0.488	0
L23	28/5/02	10.1	15.1	38.7	38.4	15.0	39	10.2	0	6.12	3.162	0.306	0.51	0.102
L24	28/5/02	7.78	14	36.4	46.8	18	38.6	8.72	0	5.494	2.442	0.262	0.436	0.087
L25	29/5/02	8.61	12	33	38.3	14	36.5	5.53	0	2.654	2.046	0.055	0.719	0.055
L26	29/5/02	8.44	11.1	30.1	35.6	13.2	37	11.8	0	5.664	5.546	0.118	0.472	0
L27	29/5/02	8.01	12.8	33.8	42.1	16	37.9	13.9	0	5.977	7.089	0.417	0.417	0
L28	29/5/02	7.5	12.7	33	44	16.9	38.4	11.5	0	5.635	5.175	0.115	0.46	0.115
L29	29/5/02	7.35	12.9	33.1	45	17.5	38.8	9.1	0	5.551	3.276	0.182	0.091	0
L30	29/5/02	9.81	14.8	38.9	39.6	15.1	38.1	8.76	0	5.081	2.978	0.088	0.613	0
L31	29/5/02	8.73	14.5	36.4	41.7	16.6	39.7	12.2	0	4.636	6.71	0.244	0.61	0
L32	29/5/02	7.42	11.8	29	39.1	15.8	40.6	6.18	0	2.781	3.028	0.062	0.247	0.062
L33	29/5/02	7.51	12.8	32.5	43.3	17	39.3	13.5	0	2.565	10.8	0.135	0	0
L34	29/5/02	9.52	13.7	35.5	37.4	14.4	38.4	11.3	0	5.085	4.859	0.226	1.017	0.113
L35	29/5/02	8.05	13	32.7	40.6	16.1	39.6	9.03	0	2.619	5.508	0.09	0.813	0
L36	29/5/02	8.75	13.6	35.2	40.2	15.5	38.5	8	0	4.64	2.8	0	0.56	0